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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

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TITLE OF INVENTION (280 characters max)

CATALYTIC ANTIBODIES AND ELECTROPHILIC ANTIGEN ANALOGS

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification (including claims and Abstract)	80 pages	☐ Small Entity Statement ☒ Small Entity Status is hereby claimed	
☒ Drawing(s)	26 Sheets 26 Figures	☐ Other (specify)	

METHOD OF PAYMENT (check one)

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Respectfully submitted,

SIGNATURE _____ Date: January 8, 2004

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PROVISIONAL APPLICATION FILING ONLY

PATENT APPLICATION SERIAL NO. _____

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Provisional Application

Catalytic Antibodies and Electrophilic Antigen Analogs

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Field of the Invention

Preparation of high-turnover catalytic antibodies, including IgM and IgG antibodies to various polypeptides.

Introduction

The invention is based on the discovery that IgM antibodies display high-level proteolytic activity. IgM preparations from immunologically naïve mice and healthy humans have been shown to cleave model peptide substrates as well as large polypeptide substrates. Monoclonal IgM antibodies display similar activities. This activity can be attributed to two factors: (a) the minimally mutated status of IgM V domains compared to their germline configuration counterparts; and (b) the unique constant domain structure of IgM.

The present invention provides novel compositions and methods for stimulating production of catalytic IgM antibodies and fragments thereof. Catalytic IgM antibodies with specificity for target antigens are therapeutically valuable. Provided herein are improved methods for identifying, isolating and refining catalytic IgM antibodies for the treatment of a variety of medical diseases and disorders, including but not limited to infectious, autoimmune and neoplastic diseases. Such catalytic antibodies will also have applications in the fields of veterinary medicine, industrial and clinical research and dermatology.

The HIV coat protein gp120 is cleaved specifically and efficiently by virtue of its recognition as a superantigen by IgM antibodies present in the preimmune repertoire. A superantigen is a molecule that is bound by certain antibody families expressed in the preimmune repertoire without the need of adaptive maturation of the antibody variable domains. Superantigen recognition by antibodies generally entails contacts at the conserved framework regions of the antibodies. The antibodies described in the present invention recognize a conserved peptide determinant of gp120 corresponding to a component of the site that binds host cell CD4 receptors and initiates infection. A covalently reactive peptide analog (CRA) of these residues described by us previously (Taguchi, H. et al Bioorg. Med. Chem. Lett. 12:3167–3170, 2002) is bound irreversibly by all IgM antibodies at levels greater than a hapten CRA devoid of the peptide. The catalytic IgM antibodies neutralize HIV, suggesting that they represent defense enzymes against HIV. Immunization with a CRA analog of full-length gp120 (Paul, S. et al. J. Biol. Chem. 278:20429-20435, 2003) resulted in synthesis of specific, irreversibly binding IgM antibodies at unusually large levels. It appears that the normal isotype switching mechanism precluding the synthesis of specific IgM Abs at high levels is precluded by CRA immunization. Co-immunization with the bacterial superantigen

protein A accentuates this effect.

Also disclosed are novel covalently reactive antigen analogs with water binding activity (CRAWs) that circumvent certain limitations in hitherto available methods for induction of catalytic antibodies. The CRAWs incorporate a metal binding site that coordinates water molecules within the antigen analogs in addition to electrophilic groups that bind covalently to the antibody nucleophiles. The presence of the bound water ensures that antibodies raised by immunization with CRAWs contain active sites can accommodate water along with the antigen substrate. This feature allows the water molecule to attack the acyl-antibody complex formed by the reaction between the antibody nucleophile and the substrate antigen. As water attack and hydrolysis of the acyl-antbody complex can be a rate limiting step in catalysis, certain antibodies to CRAWs can more efficiently complete the catalytic cycle compared to antibodies raised to the antigen and other types of antigen analogs.

This invention permits: (a) isolation of catalytic antibodies from the preimmune repertoire that are more potent than those described previously; (b) isolation of catalytic antibodies by immunization with ordinary immunogens and CRA derivatives, covalently reactive transiton state analogs (CRTSAs) and covalently reactive analogs with water binding activity (CRAWs) superior to those described previously. These analogs are collectively designated EIAs hereafter (electrophilic intermediate analogs). In addition:

1. Polyclonal and monoclonal IgM antibodies that cleave amide and peptide bonds have been identified.
2. Specific IgM antibodies that cleave gp120 at a conserved peptide epitope have been identified. These have been shown to neutralize HIV.
3. Immunization with gp120-CRA has been shown to result in robust IgM responses exceeding normal IgM responses.
4. Cleavage of CD4, EGFR and albumin by certain IgM antibodies has been demonstrated. Phage displayed lupus Fv and light chain libraries from lupus patients have been generated.
5. B cells expressing IgM on their surface have been shown to form covalent adducts with CRAs.

Summary
According to one aspect of the invention, methods and compositions are provided for stimulating catalytic antibody production to predetermined target antigens, including but not limited to those involved in pathogenic and neoplastic processes. Certain EIA formulations are useful to stimulate the production of catalytic IgM antibodies. The design principles and general structure of EIAs are shown in Figs 1 and 2. EIAs include covalently reactive analogs (CRAs; an examples is shown in Fig 3), covalently reactive analogs with water binding activity (an example is shown in Fig 4), and covalently reactive transition state analogs (CRTSAs; see Fig 5A, right top structure). The catalytic antibodies have therapeutic value in the treatment of a variety of medical conditions, including autoimmunity disorders, microbial diseases, lymphoproliferative disorders and cancer. The catalytic antibodies of the invention may also be used prophylactically to

prevent certain medical disorders, including but not limited to septic shock, systemic inflammatory disease and acute respiratory distress syndrome.

CRAs and CRTSAs have been described in previous regular and provisional U.S. patent applications and filings (US Patent 6,235,714; US Patent Application 20030078203, Covalently reactive transition state analogs and methods of use thereof, April 24, 2003; Provisional US Patent Application, Specific HIV gp120 cleaving antibodies induced by covalently reactive analogs of gp120 and methods of use thereof, filed March 26, 2003; Provisional US Patent Application, Covalent attachment of ligands to nucleophilic proteins guided by noncovalent binding; filed March 26, 2003).

CRAWS of the present invention contain a water binding site in addition to incorporating the one or more features of CRAs and CRTSAs.

EIAs with the general formula shown in Fig 2 have the following elements: (a) A nucleophile such as phosphonate diester in the case of the example CRA shown in Fig 3 and a phosphonate monoester in the case of the example CRTSA shown in Fig 5. CRAWs can contain either a phosphonate diester or a monoester. The electrophile binds antibody nucleophiles covalently. Electron withdrawing or electron donating substituents are incorporated as shown in Fig 5 to decrease or increase the electrophilic reactivity. (b) In the embodiment as the CRAW example structure shown in Fig 4, a metal binding site is located in proximity to the electrophile. (c) In the case of CRTSAs, the negatively charged oxygen atom binds noncovalently to the oxyanion hole present in certain antibodies. (d) Components of the antigen epitope are incorporated in the EIAs to allow noncovalent binding to the antibody paratope. These epitope components can be several contiguous amino acids, or, in the case of a conformational epitope, several residues that are distant from each other in the linear sequence but are spatial neighbors. Fig 6 shows as example of a linear peptide EIA as a CRA embodiment. Optionally, a mimic of the amino acid side chain corresponding to the P1 position recognized by the antibodies (first amino acid on the N-terminal side of the reaction center; e.g., amidino group mimic of Arg or Lys residues) is located close to the electrophile.

In one aspect of the invention, EIAs are administered to a living organism under conditions whereby the EIAs stimulate production of specific catalytic antibodies, including IgM and IgG antibodies. The catalytic antibodies are then purified. Antibodies so purified are then administered to a patient in need of such treatment in an amount sufficient to inactivate antigens associated with a predetermined medical disorder.

According to another aspect of the invention, a method is provided for treating a pathological condition related to the presence of endogenously expressed catalytic antibodies, including IgM and IgG antibodies. Examples of such abnormal pathological conditions are certain autoimmune disorders as well as lymphoproliferative disorders. The method comprises administering to a patient having such a pathological condition a pharmaceutical preparation comprising an EIA capable of irreversibly binding the endogenously produced catalytic antibodies, in an amount sufficient to inhibit the activity of the antibodies, thereby alleviating the pathological condition. As described in Plaque et

al (J Biol Chem. 2003 May 30;278(22):20436-43), non-catalytic antibodies also express nucleophilic sites. Therefore, the EIAs can also be employed to bind and inhibit non-catalytic pathogenic antibodies, including IgM and IgG antibodies.

According to another aspect of this invention, a pharmaceutical preparation is provided for treating a pathological condition related to the presence of endogenously produced catalytic antibodies, including IgM and IgG antibodies. This pharmaceutical preparation comprises EIAs in a biologically compatible medium. Endogenously produced catalytic antibodies are irreversibly bound and inactivated upon exposure to the EIAs. The preparation is administered an amount sufficient to inhibit the activity of the catalytic antibodies.

In another aspect of the invention, methods for passively immunizing a patient with a catalytic antibody preparation are provided. Catalytic antibodies are infused into the patient which act to inactivate targeted disease associated antigens.

In an alternative embodiment, should the patient experience unwanted side effects, the activity of the infused catalytic antibodies may be irreversibly inactivated by administering the EIAs to said patient.

In another aspect of the invention, active immunization of patients is achieved by administering the EIAs of the invention in an EIA-adjuvant complex to a patient to be immunized. One or more subsequent booster injections of the EIA-adjuvant complex at 4 week intervals can also be adminstered. Following this procedure, the patient sera will be assessed for the presence of prophylactic catalytic antibodies.

A further aspect of the invention comprises methods for screening phage displayed antibodies or B cells for expression of catalytic antibodies, including IgM antibodies. In this embodiment, phage or B cells are screened with an EIA and those phage or B cell which bind the EIA are isolated and characterized further. Methods for isolating and cloning the DNA encoding catalytic antibodies from phage or B cells so isolated are also within the scope of the present invention.

Finally, catalytic Fv fragments of antibodies and light chain components of antibodies, including IgM antibodies are also with encompassed within the present invention.

The antibodies, EIAs and methods of the present invention provide notable advantages over currently available compounds and methods for stimulating catalytic antibodies specific for predetermined target antigens. Accordingly, the disclosed compounds and methods of the invention provide valuable clinical reagents for the treatment of disease.

DESCRIPTION OF FIGURES

Fig 1: Electrophilic intermediate analogs (EIAs) are derivatives of proteins and polypeptides with the following components and properties. E1, E2, ···En and E1', E2', ··· En' are the component amino acids of the antigenic epitope recognized noncovalently

by the antibody. The noncovalent interactions occur in conjunction with covalent interaction between one or more antibody nucleophile (Nu) and one or more EIA electrophile Y. E1-En and E-'-En' can be a linear or discontinuous set of amino acids that are spatially in proximity with electrophile Y. Dotted lines connecting E1-En and E'-En' can represent short or extended lengths of the polypeptide backbone that do not serve as components of the antigenic epitope. As proteins can express one or more antigenic epitope, the EIA may contain one or more sets of each reactive unit composed of E1-En and E1'-En'. Optionally, Y can contain a peptide extension, which is also capable contributing noncovalent contacts. A metal binding site is placed in proximity to Y, which structure serves as an example CRAW embodiment of EIAs. Examples of metals that can be coordinated by the metal binding site are zinc, copper, nickel, calcium, manganese and magnesium. In turn, the bound metals coordinate water molecules. Proximity of water to Y is intended to ensure that antibodies induced by immunization with EIA contain space in their active site into which water can diffuse. The availability of water enhances the probability of completion of the catalytic cycle, which entails hydrolysis of the covalent acyl-antibody intermediate. In the extreme case, such antibodies might also be capable of binding metals that can coordinate water, but this is not a mandatory requirement. Y can be linked to one or more amino acid side chains of the protein or it can be placed within the peptide backbone. In the latter case, the phosphorus atom mimics the carbon atom and the P-O bond mimics the peptide bond of polypeptides. Examples of the site of linkage of Y to amino acid side chains include the –NH2, -COOH, -SH and -OH groups found in Lys, Asp, Glu, Cys, Ser, Thr and Tyr. Y can include an uncharged phosphonate diester or a negatively charged phosphonate monoester. These compounds are example CRA and CRTSA embodiments of EIAs. Linkage of Y to amino acid side chain can be accomplished directly or through the use of an adaptor functionality.

FIG. 2: **General structure of EIAs and two examples of X-X'-Y substituents.** Y is an electrophilic group. Optionally, Y can contain peptide extensions. X' is a chemical bond, adaptor group or a metal binding moiety. X is a functional group of R2 to which X' is attached. R2 is a component unit of the ligand such as an amino acid, sugar residue or fatty acid group. R1 and R3 are chemical groups that are located in the spatial neighborhood of Y and contribute one or more sites capable of noncovalent binding to proteins.

Fig 3. Schematic structure of an example protein CRA, a subgroup of EIAs.

Fig 4: Schematic structure of an example protein CRAWs, a subgroup of EIAs.

Fig 5: Examples of Y group variants. In CRAs,Y contains the uncharged phosphonate diester that serves as the electrophile. In CRTSAs, Y contains the phosphonate monoester group, which presents a negative charge in addition to the electrophilic phosphorus atom. CRAWs contain either type of Y group (phosphonate diester or phosphonate diester). Depending on the nature of R3 and R4, the covalent reactivity is increased (A) or decreased (B). Peptide extensions can be included in R3 or R4 as shown in (C).

Fig 6. Example of a linear peptide CRA in which Y provides noncovalent binding interactions with proteins (Met-Trp-Gln-Glu-Leu-Gly-AmP-Ala-Met-Tyr; AmP, amidinophosphonate mimic of Arg).

Fig 7. Examples of metal binding sites incorporated in EIAs. These sites can bind metals such as Ni, Zn, Cu, Mn, Ca and Mg. Metal binding is monitored by methods such as atomic absorption spectrometry. Bound water is determined by X-ray crystallography.

FIGS. 8A and 8B. A list of antigens targeted by conventional monoclonal antibodies showing clinical promise. Such antigens are suitable targets for the catalytic antibodies, including IgM antibodies of the present invention.

DESCRIPTION OF ATTACHED MANUSCRIPTS

The manuscript attached hereto entitled *Ontogeny of Proteolytic Immunity:IgM Serine Proteases* (Planque et al.), its Figures 1 – 8, and Tables 1 and 2, are part of this provisional application.

The manuscript attached hereto entitled *Selective IgM-Catalyzed Hydrolysis of HIV gp120: An innate defense against gp120?* (Karle et al.), its Figures 1 – 7, and Tables 1 and 2, are also part of this provisional application.

Further Notes Relevant to the Invention

Methods are disclosed for stimulating the synthesis of catalytic antibodies, including IgM and IgG antibodies, of predetermined specificity by the immune system. The catalytic antibodies contemplated in the present invention will constitute a major improvement over such conventional monoclonal antibodies because of their ability to affect many target molecules vs. just one and because of the resulting dramatic decrease in the cost of treatment.

Polypeptides to be targeted include soluble ligands and the membrane bound receptors for these ligands. A listing of some of the antigens targeted by conventional monoclonal antibodies showing clinical promise and the corresponding medical indications are shown in FIGS. 8A and 8B. Suitable categories of prophylactic or therapeutic target peptide antigens for the practice of the present invention include but are not limited to cytokines, growth factors, cytokine and growth factor receptors, proteins involved in the transduction of stimuli initiated by growth factor receptors, clotting factors, integrins, antigen receptors, enzymes, transcriptional regulators particularly those involved in cellular program (differentiation, proliferation and programmed cell death) control, other inducers of these cellular programs, cellular pumps capable of expelling anticancer agents, microbial and viral peptide antigens.

Microbial proteins can also be targeted for catalysis by the antibodies of the present invention. These include but are not limited to gp120, gp160, Lex1 repressor, gag, pol, hepatitis B surface antigen, bacterial exotoxins (diptheria toxin, C. tetani toxin, C. botulinum toxin, pertussis toxin). An exemplary B cell epitope suitable for targeting of

HIV by catalytic antibodies is KQIINMWQEVGKAMYA. This peptide is derived from the CD4 binding site, which is generally conserved in different HIV-1 strains.

Neoplastic antigens can also be incorporated into therapeutically beneficial EIAs. These include but are not limited to EGF, TGF.alpha., p53 products, prostate specific antigen, carcinoembryonic antigen, prolactin, human chorionic gonadotropin, c-myc, c-fos, c-jun, p-glycoproteins, multidrug resistance associated proteins, metalloproteinases and angiogenesis factors.

Receptors for neoplastic antigens will also be targeted for antibody-mediated catalysis. These include EGFR, EGFR mutants, HER-2, prolactin receptors, and steroid receptors.

Inflammatory mediators are also suitable targets for catalysis. Exemplary molecules in this group include TNF, IL-1beta, IL-4 as well as their cognate receptors.

The EIAs are prepared using conventional organic synthetic schemes. The EIAs contain at least one epitope derived from proteins associated with a particular peptide antigen to be targeted for cleavage and the intended use of the EIA. Selection of linear flanking amino acid sequences and epitopes depends on the particular peptide antigen targeted for cleavage. For example, viral coat proteins, certain cytokines, and tumor-associated antigens contain many different epitopes. Many of these have been mapped using conventional monoclonal Ab-based methods and can be incorporated in the EIA structure. The structural features of the EIAs are intended to permit specific and covalent binding to immature, germline encoded antibodies as well as mature antibodies specialized to recognize the targeted epitope. Based on the tenets of the clonal selection theory, the EIAs are also intended to recruit the germline genes encoding the catalytic antibodies for the synthesis of mature antibodies directed towards the targeted epitope.

The EIAs which elicit production of the desired antibodies are composed of certain basic elements. These include an electrophilic reaction center, and at least one antigenic epitope of the target antigen. In a preferred embodiment, the electrophilic reaction center is selected from the group of molecules shown in Fig. 2. EIAs can be prepared from large proteins or synthetic peptides. The electrophilic group Y can be ,located in amino acid side chains, within the peptide backbone or at the termini of the polypeptide as desired. In the CRAW embodiment of EIAs, a water molecule is forced within spatial proximity of Y. Thus, antibodies induced by CRAW immunization not only contain a nucleophilic group capable of reacting with electrophiles, but can also accommodate water molecules in their active sites, facilitating completion of the catalytic cycle.

Covalently reactive antigen analogs (CRAA) have been described in U.S. Pat. No. 6,235,714, and U.S. Patent Application Publication 2003/0078203, which are both incorporated herein by reference.

The EIAs of the invention and the resulting catalytic antibodies have at least three major applications. The first application is directed to the generation of catalytic antibodies in either humans or animals following immunization with a EIA designed for a particular

medical disorder. The catalytic antibodies so generated would then be administered to patients to inactivate targeted antigen moieties. In this scenario, should the patient experience adverse side effects, the immunizing EIA may be administered to irreversibly inactivate the catalytic antibody. The EIA in this embodiment would be synthesized with a B cell epitope only in order to minimize immunogenicity.

In the second application, EIAs may be administered to patients for the purposes of actively immunizing the patient against particular pathological to generate a state of protective immunity. These EIAs would be administered as an EIA-adjuvant complex.

In the third application, the EIAs of the invention may be administered to patients who are currently expressing pathogenic catalytic antibodies in association with a medical disorder such as autoimmune disease or multiple myeloma. EIAs may be designed to specifically react with the pathogenic antibodies. Inhibition of catalytic function should result in an amelioration of the disease state. Again, these EIAs may be designed to minimize their immunogenicity.

In one embodiment of the invention, vaccination protocols are described which elicit catalytic Ab production to predetermined viral or pathogenic antigens. The EIA immunogens disclosed preferentially stimulate the production of catalytic antibodies, from which IgM, IgG and IgA antibodies can be isolated. Such antibodies provide superior protection against infection due to the presence of catalytic action against the target antigen which results in its permanent inactivation. Additionally, a single catalytic Ab molecule may be reused to inactivate multiple antigen molecules as compared to noncatalytic Abs which bind antigen reversibly and stoichiometrically. Active immunization will be done using previously developed methods with vaccines designed to elicit protective IgM antibody responses against the desired antigens For example, the EIAs mixed with a suitable adjuvant formulation such as alum can be administered intramuscularly at a dose optimized for maximum antibody synthesis, and two or three booster injections can be administered at 4 week intervals, until the catalytic antibody concentration in the serum reaches plateau levels. The protective immunity so generated is anticipated to last for several years, because vaccination will result in formation of specific, long lived memory cells that can be stimulated to produce antibodies upon exposure to the offending organism or cancer cell. Because antibody synthetic response to most antigens are T cell dependent, an appropriate T cell epitope can be incorporated into the immunogen by peptide synthesis. Alternatively, a carrier such as keyhole limpet hemocyanin can be conjugated to the EIA via coupling through lys side chain amino groups or Cys side chain SH groups to maximize the antibody response if necessary.

EIAs as described herein are generally administered to a patient as a pharmaceutical preparation. The term "patient" as used herein refers to human or animal subjects. The pharmaceutical preparation comprising the EIAs of the invention are conveniently formulated for administration with a acceptable medium such as water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), dimethyl sulfoxide (DMSO), oils, detergents, suspending agents or suitable mixtures thereof. EIAs may be administered parenterally by intravenous injection into

the blood stream, or by subcutaneous, intramuscular or intraperitoneal injection. Pharmaceutical preparations for parenteral injection are commonly known in the art. If parenteral injection is selected as a method for administering the molecules of the invention, steps must be taken to ensure that sufficient amounts of the molecules reach their target cells to exert a biological effect.

Conventional immunization methods will applied to induce catalytic Ab synthesis. One or more injections of the immunogen will be administered. RIBI will be used as adjuvant in the animal studies. For human use, alum will be employed as the adjuvant. Alum is approved for human use. Analysis of multiple adjuvants is advantageous because the quality and magnitude of Ab responses to vaccines can be influenced by adjuvants, via effects of the cytokines and TH subpopulations recruited by the adjuvants on B

In addition to the vaccination approach described above, monoclonal antibodies with catalytic activity are used for passive immunotherapy applications. For this purpose, the antibodies can be cloned by hybridoma technology or they can be isolated by display methods such as phage display techniques. Mutagenesis is conducted to improve catalytic efficiency and specificity, using in vitro antibody maturation techniques. The catalytic antibodies described herein include the different Ig class and subclasses, e.g., IgG, IgM and IgA. CRAs and CRTSAs have previously been applied to select catalysts from the antibody libraries, and CRAW selection procedures should yield even better catalysts.

Conventional passive immunization methods will be employed when administering the catalytic antibodies of the invention. In a preferred embodiment, Abs will be infused intravenously into the patient. For treatment of certain medical disorders, steps must be taken to ensure that sufficient amounts of the molecules reach their target cells to exert a biological effect. The antibodies are administered to a patient as a pharmaceutical preparation formulated for administration with an acceptable medium such as water, buffered saline, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), dimethyl sulfoxide (DMSO), oils, detergents, suspending agents or suitable mixtures thereof.

The lipophilicity of the molecules, or the pharmaceutical preparation in which they are delivered may have to be increased so that the molecules can arrive at their target locations. Furthermore, the catalytic antibodies of the invention may have to be delivered in a cell-targeted carrier so that sufficient numbers of molecules will reach the target cells. Methods for increasing the lipophilicity and targeting of therapeutic molecules, which include capsulation of the catalytic antibodies of the invention into antibody studded liposomes, are known in the art.

The catalytic antibodies that are the subject of the present invention can be used as antibody fragments or whole antibodies or they can be incorporated into a recombinant molecule or conjugated to a carrier such as polyethylene glycol. In addition any such fragments or whole antibodies can be bound to carriers capable of causing the transfer of said antibodies or fragments across cell membranes as mentioned above. Carriers of this

type include but are not limited to those described (Cruikshank et al. in the Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology, March 1997).

The pharmaceutical preparation is formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to a physically discrete unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art. For example, the half-life of syngeneic IgG in the human is about 20 days. Over this period, 60,480 Ag molecules will be cleaved by one molecule of an antibody with a turnover of 2.1/min (which is the turnover of a human anti-VIP L chain isolated from a phage display library. It can be seen, therefore, that the peptidase antibodies can express considerably more potent antigen neutralizing activity than stoichiometric, reversibly-binding molecules.

The pharmaceutical preparation comprising the catalytic antibodies may be administered at appropriate intervals, for example, twice a week until the pathological symptoms are reduced or alleviated, after which the dosage may be reduced to a maintenance level. The appropriate interval in a particular case would normally depend on the condition and the pathogenic state sought to be treated in the patient.

There are many areas in medicine where monoclonal antibody administration is providing clinical benefit. In the field of organ transplantation, a MoAb (OKT3) which binds to the T cell receptor has been employed to deplete T cells in vivo. Additionally, MoAbs are being used to treat graft v. host disease with some success. Accordingly, methods of administration of monoclonal antibodies are well known to clinicians of ordinary skill in the art.

Following infusion, patients will be assessed to determine the efficacy of the administered catalytic antibody. Should the catalytic antibodies administered as above give rise to undesirable side effects in the patient, the immunizing EIAs will be administered to covalently inhibit the action of the catalytic antibodies.

Catalytic Ab synthesis occurs spontaneously in autoimmune and lymphoproliferative diseases. These catalytic antibodies are thought to be important in disease pathogenesis. EIAs are capable of inactivating the catalytic antibodies and ameliorating the the pathogenic antibody effects. The harmful actions of these catalytic antibodies will be inhibited by administering EIAs to patients. EIAs designed to be weakly immunogenic will be administered. Examples of EIAs used for this purpose are those derived from VIP, Arg-vasopressin, thyroglobulin, thyroid peroxidase, IL-1, IL-2, interferons, proteinase-3, glutamate decarboxylase

The pharmaceutical preparation is formulated in dosage unit form for ease of The pharmaceutical preparation comprising the EIA may be administered at appropriate intervals, for example, twice a day until the pathological symptoms are reduced or

alleviated, after which the dosage may be reduced to a maintenance level. The appropriate interval in a particular case would normally depend on the condition and the pathogenic state sought to be treated in the patient.

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

Supporting information: Group 1 Claims:

Claims 5,6: Polyclonal IgM antibodies purified from HIV-1 infected subjects were found to cleave gp120 as shown in the following table.

IgM code	gp120 cleavage, %
5	82.4
4	72.2
7	66.4
10	45.0
11	0.8
12	57.6

IgM purified by affinity chromatography on immobilized anti-IgM antibody from sera of six subjects positive for HIV infection. Reaction conditions, IgM 25 nM; biotinylated gp120 100 nM; 20 hours incubation. Cleavage determined by densitometry of SDS-electrophoresis gels (depletion of intact gp120 band).

Claim 7: Polyclonal IgM antibodies purified from patients with systemic lupus erythematosus were observed to cleave gp120 as shown in the following table.

IgM code	gp120 cleavage, %
1017	40.5
1018	62.3
1019	49.3
1020	42.7
1063	34.4

IgM purified by affinity chromatography on immobilized anti-IgM antibody from sera of six subjects positive for HIV infection. Reaction conditions, IgM 25 nM; biotinylated gp120 100 nM; 20 hours incubation. Cleavage determined by densitometry of SDS-electrophoresis gels (depletion of intact gp120 band).

Claim 8-10: Following treatment of biotinylated soluble CD4 (Bt-sCD4; NIH AIDS Reagent Repository), soluble epidermal growth factor receptor (Bt-sEGFR) and bovine

serum albumin (Bt-BSA) with polyclonal human IgM (1 μM IgM, 15 hours; 100 nM substrate, product bands of Bt-sCD4 (Mr 12-18 kD), Bt-sEGFR (32 kD) and Bt-BSA (28-44 kD) were visible.

Claim 11: Synthesis of human antibodies is induced by immunization of transgenic mice expressing human antibodies genes (Curr Opin Biotechnol. 2002 Dec;13(6):593-7. Antibody discovery: the use of transgenic mice to generate human monoclonal antibodies for therapeutics. Kellermann SA, Green LL).

Claim 12: The VL and VH genes of monoclonal IgM antibodies is routinely cloned and sequenced from hybridoma cells or recombinant antibody repertoires by techniques such as reverse transcriptase-polymerase chain reaction and dideoxy nucleotide sequencing (J Biol Chem. 1994 Dec 23;269(51):32389-93. Molecular cloning of a proteolytic antibody light chain. Gao QS, Sun M, Tyutyulkova S, Webster D, Rees A, Tramontano A, Massey RJ, Paul S.; J Biol Chem. 2001 Jul 27;276(30):28314-20. Phosphonate ester probes for proteolytic antibodies. Paul S, Tramontano A, Gololobov G, Zhou YX, Taguchi H, Karle S, Nishiyama Y, Planque S, George S).

Claim 13: The VL and VH domains are cloned into previously described expression vectors for production as full-length antibodies. The vectors contain constant domains corresponding to different Ig class and subclass, e.g., IgG, IgM IgA and IgE (e.g., US patents 6,407,213 and 5,807,715).

Claims 14,15: The IgM antibodies are raised by immunization with CRAs, similar to previously described protocols for raising IgG antibodies by immunization with CRAs (J Biol Chem. 2003 May 30;278(22):20429-35. Specific HIV gp120-cleaving antibodies induced by covalently reactive analog of gp120. Paul S, Planque S, Zhou YX, Taguchi H, Bhatia G, Karle S, Hanson C, Nishiyama Y). Examples of hapten and polypeptide CRAs are described in J Biol Chem. 2001 Jul 27;276(30):28314-20. Phosphonate ester probes for proteolytic antibodies. Paul S, Tramontano A, Gololobov G, Zhou YX, Taguchi H, Karle S, Nishiyama Y, Planque S, George S; Arch Biochem Biophys. 2002 Jun 15;402(2):281-8. Covalent reactivity of phosphonate monophenyl esters with serine proteinases: an overlooked feature of presumed transition state analogs. Nishiyama Y, Taguchi H, Luo JQ, Zhou YX, Burr G, Karle S, Paul S; J Biol Chem. 2003 May 30;278(22):20436-43. Broadly distributed chemical reactivity of natural antibodies expressed in coordination with specific antigen binding activity. Planque S, Taguchi H, Burr G, Bhatia G, Karle S, Zhou YX, Nishiyama Y, Paul S). Rapid assay of large numbers of IgM samples is achieved by covalent ELISA and purification of the antibodies for study catalysis.

Claim 16: IgM antibodies can be raised by in vitro methods such as are described previously (e.g., J Immunol Methods. 1997 Jan 15;200(1-2):181-90. Induction of antigen-specific isotype switching by in vitro immunization of human naive B lymphocytes. Zafiropoulos A, Andersson E, Krambovitis E, Borrebaeck CA.

Supporting Information: Group 2 claims
Induction of specific catalytic IgM and IgG antibodies can be employed for prophylactic purposes. The overall procedures are similar to those employed to induce the synthesis of IgG antibodies, i.e., use of covalently reactive analogs of various antigens (J Biol Chem. 2003, 278:20429-35. Specific HIV gp120-cleaving antibodies induced by covalently reactive analog of gp120. Paul S, Planque S, Zhou YX, Taguchi H, Bhatia G, Karle S, Hanson C, Nishiyama Y). The technology is applicable to microbial antigens such as HIV gp120, tumor-associated antigens such as EGFR and endogenous antigens such as soluble polypeptides and proteins and cell surface receptors. Examples of the peptide and protein covalent analogs and methods for raising antibodies by immunization with the covalent analogs are described in:

Paul et al., US Patent 6,235,714 (Methods for identifying inducers and inhibitors of proteolytic antibodies, compositions and their uses May 22, 2001); incorporated herein by reference.

Paul et al Published US Patent Application 20030078203 (Covalently reactive transition state analogs and methods of use thereof; April 24, 2003); incorporated herein by reference.

Paul and Nishiyama, Provisional US Patent Application (Specific HIV gp120 cleaving antibodies induced by covalently reactive analogs of gp120 and methods of use thereof; filed March 26, 2003, incorporated herein by reference.

Paul, S. et al Provisional US Patent Application (Covalent attachment of ligands to nucleophilic proteins guided by noncovalent binding; filed March 26, 2003; incorporated herein by reference.

Supporting Information: Group 3 claims
Like IgG autoantibodies, IgM autoantibodies are well known to contribute to the pathogenesis of autoimmune and alloimmune diseases. Inhibition of pathogenic antibodies by covalent antigen analogs is described in the patent filings listed above. In addition, we have recently published a paper documenting the feasibility of this goal (Nishiyma et al, J. Biol Chem, epub Dec 2003), incorporated herein by reference.

Claims:

Group 1 claims: Catalytic antibodies:

1. Monoclonal antibodies from humans or animals that catalyze the cleavage of amide bonds, including IgM and IgG antibodies.
2. Monoclonal antibodies from humans or animals that catalyze the cleavage of peptide bonds, including IgM and IgG antibodies.
3. Monoclonal proteolytic antibodies from humans or animals that catalyze the cleavage of superantigen polypeptides, including IgM and IgG antibodies.

4. Monoclonal proteolytic antibodies from humans or animals that catalyze the cleavage of HIV gp120, including IgM and IgG antibodies.
5. Monoclonal proteolytic antibodies from HIV-infected humans that catalyze the cleavage of superantigen polypeptides, including IgM and IgG antibodies.
6. Monoclonal proteolytic antibodies from HIV-infected humans that catalyze the cleavage of HIV gp120, including IgM and IgG antibodies.
7. Monoclonal proteolytic antibodies from humans with autoimmune disease that catalyze the cleavage of peptide bonds, including IgM and IgG antibodies.
8. Monoclonal proteolytic antibodies from patients with autoimmune disease that catalyze the cleavage of autoantigen polypeptides, including IgM and IgG antibodies.
9. Monoclonal proteolytic antibodies from patients with autoimmune disease that catalyze the cleavage of CD4, including IgM and IgG antibodies.
10. Monoclonal proteolytic antibodies from patients with autoimmune disease that catalyze the cleavage of EGFR, including IgM and IgG antibodies.
11. Monoclonal proteolytic antibodies from animals engineered to express human antibodies that catalyze the cleavage of peptide bonds, including IgM and IgG antibodies.
12. The catalytic VL and VH domains of the antibodies in claims 1-11.
13. The catalytic VL and VH domains of the antibodies in claims 1-11 expressed as IgG, IgA and IgE antibodies.
14. Monoclonal antibodies with the ability to catalyze the cleavage of peptide bonds obtained by immunization of animals with peptide antigens and their EIA derivatives.
15. Monoclonal antibodies with the ability to catalyze the cleavage of gp120 obtained by immunization of animals with gp120, EIA derivatives of gp120 and EIA derivatives of synthetic gp120 peptides.
16. Monoclonal antibodies with the ability to catalyze the cleavage of peptide bonds obtained by in vitro immunization of lymphocytes with peptide antigens and their EIA derivatives.

Group 2 claims: Methods to vaccinate against microorganisms by eliciting prophylactic catalytic immunity, including IgM-mediated catalytic immunity and IgG-mediated catalytic immunity.

17. A method for stimulating production of antibodies, including IgM and IgG antibodies having catalytic activity, comprising: a) administering to a test subject, an immunogenic amount of a covalently reactive transition state analog; b) repeating step a) as necessary to ensure effective antibody production; and c) isolating and purifying said antibodies.
18. A catalytic antibody produced by the method of claim 17.
19. A method for treating a disease state in a patient, comprising: administering a therapeutically effective amount of antibodies having catalytic activity, produced by the method of claim 17.
20. A method of inhibiting the catalytic antibody used in the treatment of claim

19, comprising: a) administering to said patient an EIA, said EIA binding said catalytic antibody irreversibly; b) assessing said patient for inhibition of catalytic antibody activity; c) repeating step a) as necessary to maintain inhibition of said catalytic antibody activity.

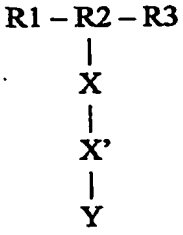
21. A method for passively immunizing a patient, comprising: a) administering to said patient a catalytic antibody specific for an antigen associated with a medical disorder diagnosed in said patient; b) repeating step a) as necessary to maintain immunity; c) assessing said patient's sera for the presence of catalytic antibodies.
22. A method for actively immunizing a patient, against a microbial infection, comprising: a) complexing an EIA comprising an immunogenic microbial epitope from an infectious organism with an adjuvant, said EIA-epitope-adjuvant complex comprising a vaccine. Deleted because text is unnecessarily restrictive.

Group 3 claims: Methods to inhibit pathogenic catalytic autoantibodies, including IgM and IgG autoantibodies found in autoimmune disease using CRA derivatives of autoantigens.

23. A method of treating a disease state in a patient by irreversibly inhibiting the action of a catalytic antibody comprising: a) administering to said patient a therapeutic amount of an EIA, said EIA comprising an epitope recognized and irreversibly bound by said catalytic antibody; b) assessing said patient for inactivation of said catalytic antibody; and c) repeating step a) as necessary to maintain inhibition of said action of said catalytic antibody.
24. A method as claimed in claim 23, wherein said disease state is an autoimmune disease.
25. A method as claimed in claim 24, wherein said autoimmune disease is selected from the group consisting of autoimmune thyroiditis, systemic lupus erythmatosus, asthma, rheumatoid arthritis, mixed connective disease, Reiter's syndrome, Sjogren's syndrome, vasculitis, and bird shot retinopathy.
26. A method as claimed in claim 23, wherein said disease state is a lymphoproliferative disorder.
27. A method as claimed in claim 26, wherein said lymphoproliferative disorder is selected from the group consisting of multiple myeloma, acute lymphoblastic leukemia, lymphoblastic- lymphoma, Small lymphocytic lymphoma, lymphoplasmacytoid lymphoma, Waldenstroms macroglobinemia, Follicular Center, lymphoma, mucose-associated lymphoid tissue lymphoma, Hairy Cell Leukemia, Diffuse Large B-Cell lymphoma, Burkitts Lymphoma, and Node based moncocytoid lymphoma.

Group 4 claims:

28. A compound of formula



wherein Y is an electrophilic group, optionally containing peptide extensions,

X' is a chemical bond, adaptor group or a metal binding moiety,

X is a functional group of R2, which is a component of the ligand such as an amino acid, sugar residue or fatty acid group,

R1 and R3 are chemical groups that are located in the spatial neighborhood of Y and contribute one or more sites capable of noncovalent binding to proteins.

29. The EIAs of claim 28 which contains a water binding site.

30. The EIA of claim 29, which contains a cyclic polyamine moiety.

30. A method for inhibiting non-catalytic antibodies, comprising: contacting an EIA of clam 28 with a non-catalytic antibody that binds to said EIA.

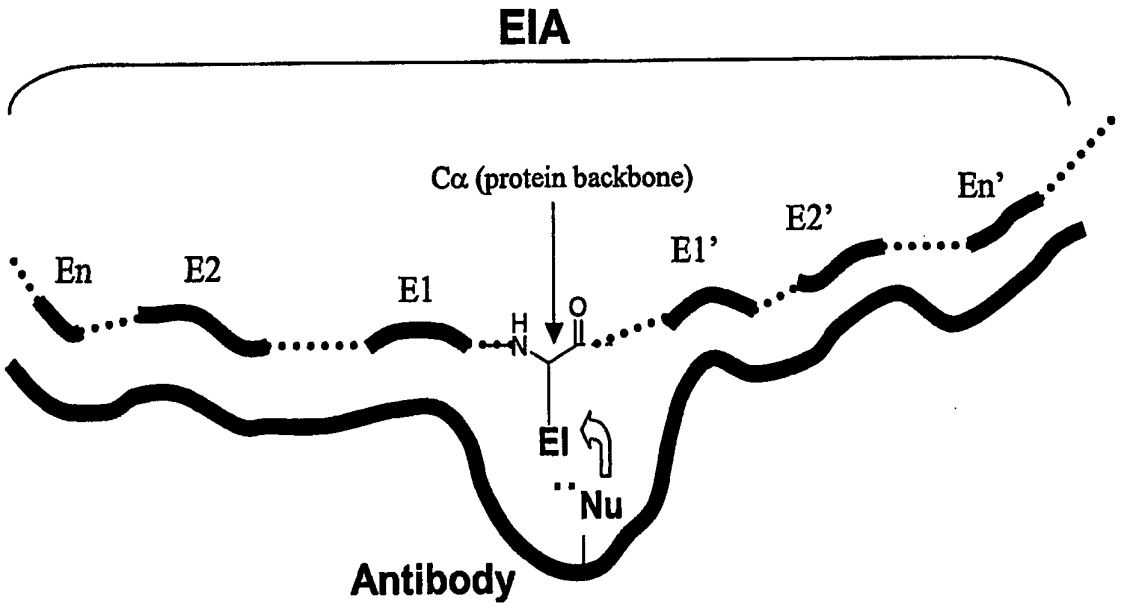
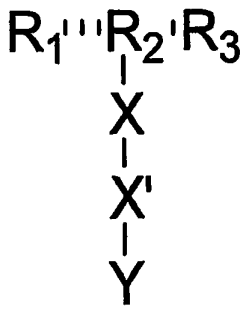


Fig 1

General EIA structure

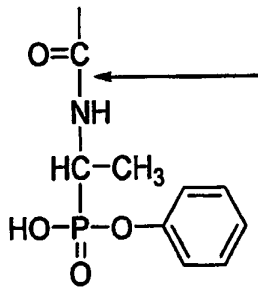


X-X'-Y, Example 1

X = β-carboxyl group of Asp

X' = amide bond

Y = monophenyl 1-aminoethanephosphonate



X-X'-Y, Example 2

X = ε-amino group of Lys

X' = suberic acid residue

Y = diphenyl amino(4-amidinophenyl)-methanephosphonate

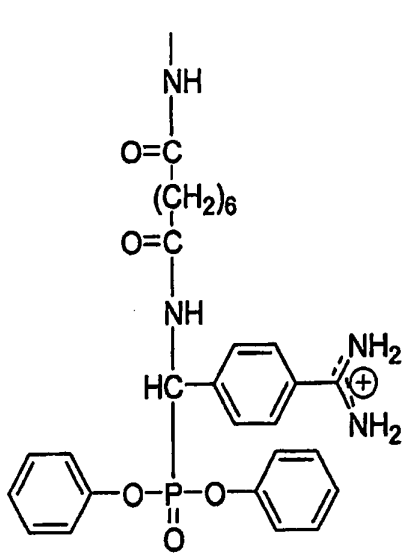
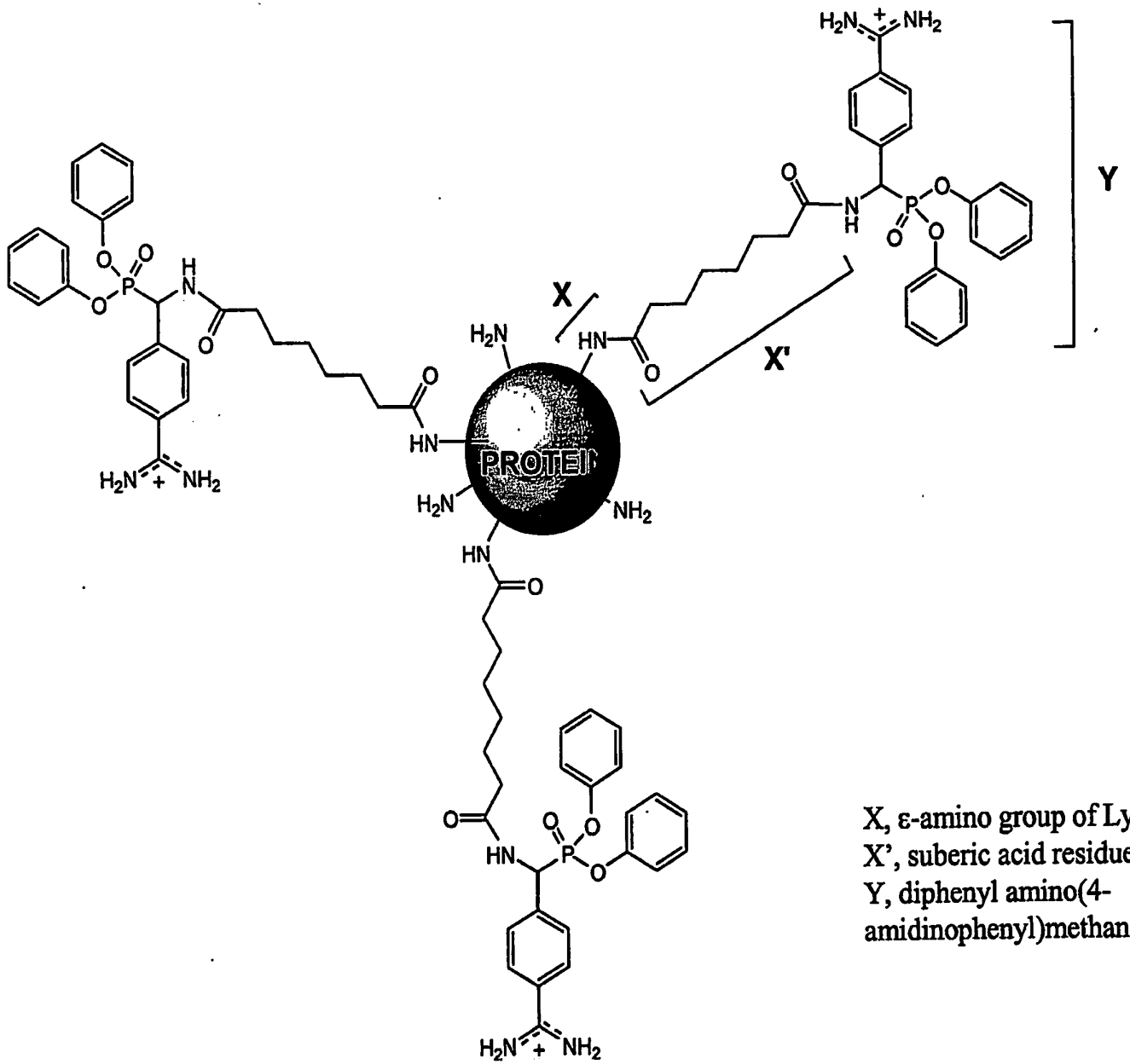


Fig. 2



X, ε-amino group of Lys
X', suberic acid residue
Y, diphenyl amino(4-amidinophenyl)methanephosphonate

Fig. 3

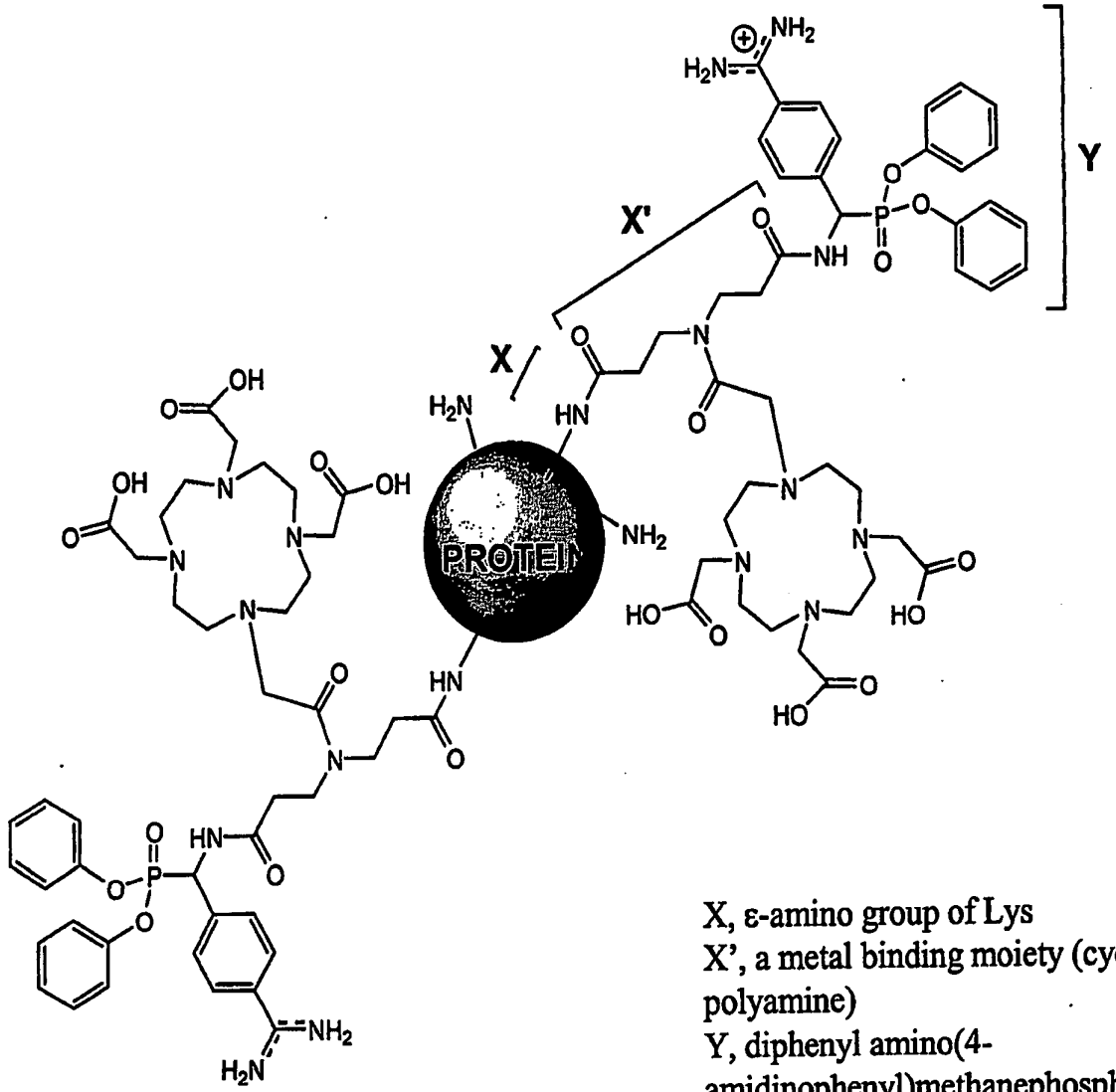

X'
X
Y
PROTEIN
X, ε-amino group of Lys
X', a metal binding moiety (cyclic polyamine)
Y, diphenyl amino(4-amidinophenyl)methanephosphonate

Fig. 4

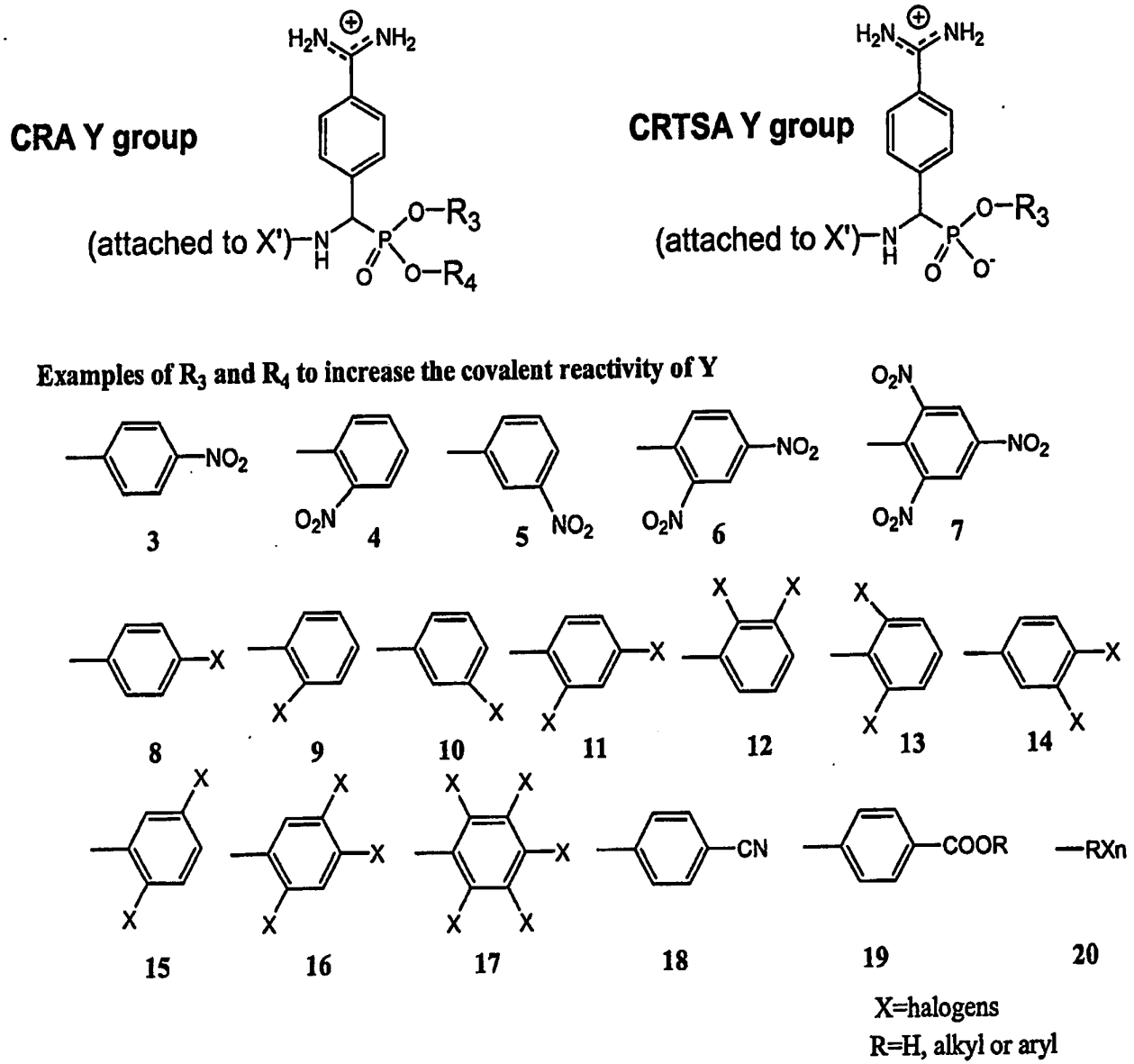
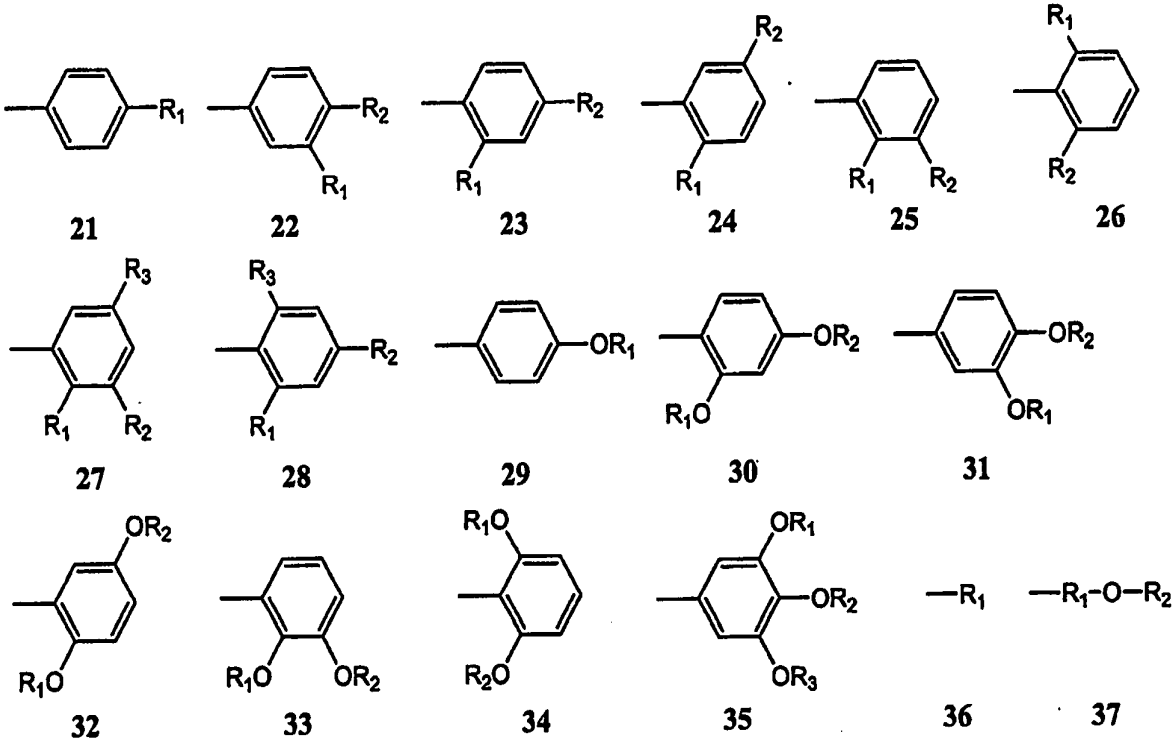

CRA Y group
(attached to X')–N(H)–P(=O)(O–R3)(O–R4)
CRTSA Y group
(attached to X')–N(H)–P(=O)(O–R3)(O-)
Examples of R3 and R4 to increase the covalent reactivity of Y
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
X=halogens
R=H, alkyl or aryl

Fig. 5 A

Examples of R_3 and R_4 to decrease the covalent reactivity of Y

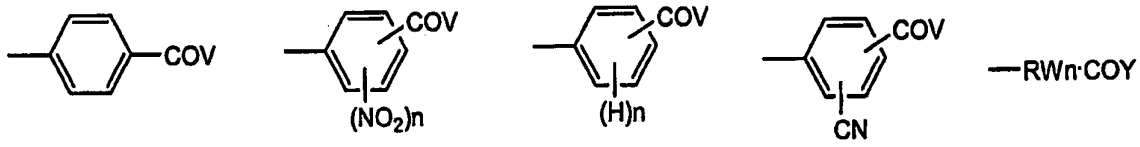


R_n= H, alkyl or aryl

Fig. 5 B

Examples of R_3 and R_4 with peptide extension

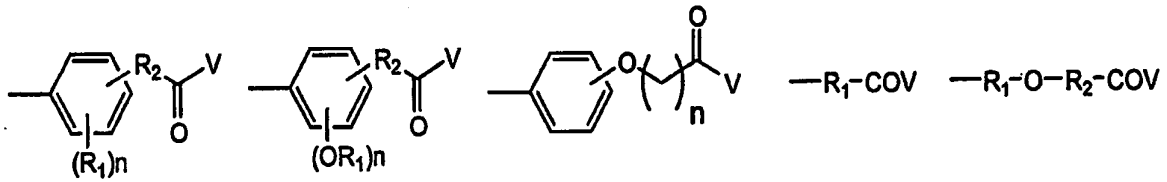
A. Electron withdrawing substituents with peptide extension



38 39 40 41 42

W: halogen
V: peptide
R: alkyl or aryl

B. Electron withdrawing substituents with peptide extension



43 44 45 46 47

V: peptide
R: alkyl or aryl

Fig. 5 C

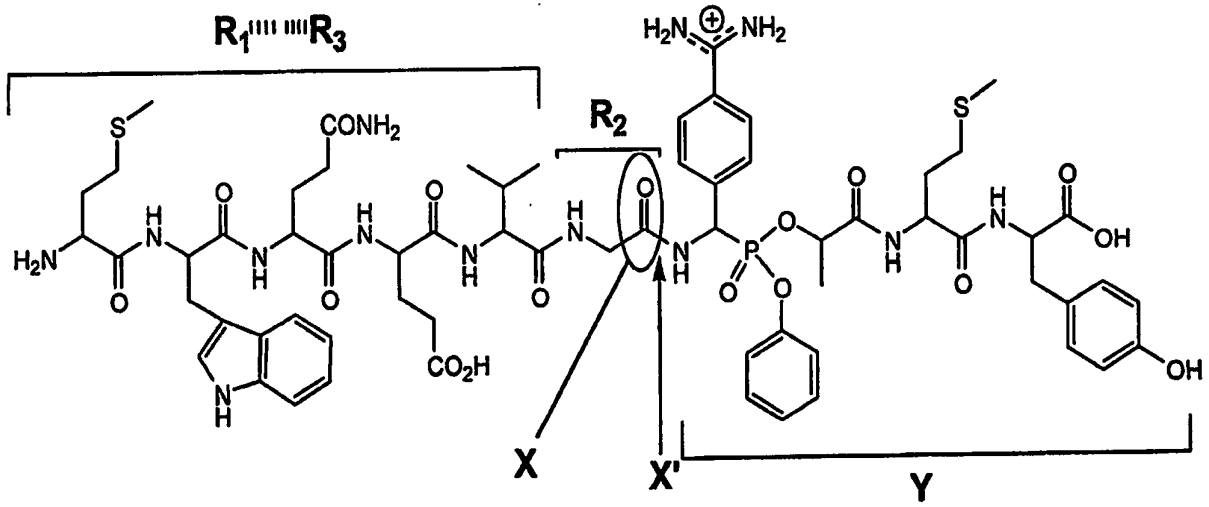
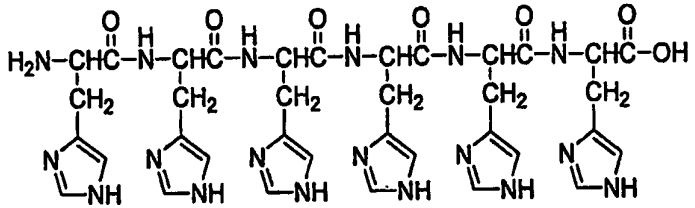


Fig. 6

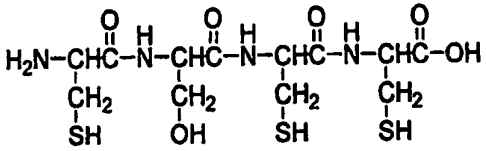
Selected examples of metal binding moiety

1. (His)n
2. (Cys-Aaa-Cys-Cys): metallothionein α-domain-derived peptide
3. (Cys-Aaa-Cys): metallothionein β-domain-derived peptide
4. EDTA
5. a crown ether

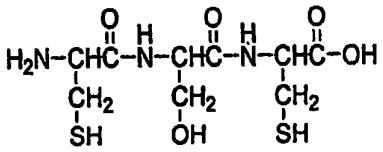
Example of 1: $(His)_6$



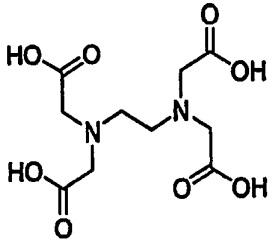
Example of 2: Cys-Ser-Cys-Cys



Example of 3: Cys-Ser-Cys



Example of 4: EDTA



Example of 5: 4'-Carboxybenzo-18-crown-6

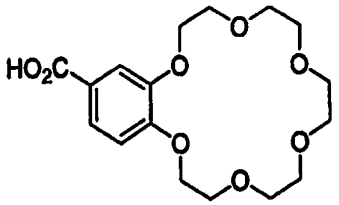


Fig 7

Target Antigen	Disease Indications
CD4	Rheumatoid Arthritis, Asthma, Transplantation, Autoimmune Disease
HER2	Carcinoma
EGFR	Carcinoma
Macrophage Inhibitory Factor	Inflammatory and Autoimmune Disease
CD80 (B7-1)	Inflammatory and Autoimmune Disease, Atherosclerosis
CD86 (B7-2)	Inflammatory and Autoimmune Disease, Atherosclerosis
CD28	Inflammatory and Autoimmune Disease, Atherosclerosis
CD70	Inflammatory and Autoimmune Disease, Atherosclerosis
CD11b/CD18	Arthritis, Inflammatory and Autoimmune Disease
CD23	Arthritis, Inflammatory and Autoimmune Disease
ICAM-1	Inflammatory and Autoimmune Disease, Rheumatoid Arthritis, Inflammatory Bowel Disease, Organ Transplant Rejection, Psoriasis, Atherosclerosis
VLA-4 Intrgrin Receptor	Inflammatory and Autoimmune Disease
TNF-alpha	Rheumatoid Arthritis, Autoimmune Disease, Neurotropic Pain, Ischemia-reperfusion Injury, Septic Shock, SIRS, ARDS, Multiple Sclerosis, AIDS
Complement Component C5	Autoimmune Disease, Immunosuppression

FIG. 8 A

Target Antigen	Disease Indications
IL-1 beta Receptor	Rheumatoid Arthritis, Autoimmune Disease, Neurotropic Pain, Ischemia-reperfusion Injury, Septic Shock, SIRS, ARDS
IL-1 beta	Rheumatoid Arthritis, Autoimmune Disease, Neurotropic Pain, Ischemia-reperfusion Injury, Septic Shock, SIRS, ARDS
GPIIb/IIIa Receptor	Anti-thrombotic, Use in combination with Angioplasty, Percutaneous Coronary Intervention, Unstable Angina, Stroke
Clotting Factor VII	Anti-coagulant
Plasminogen Activator Inhibitor (PAI-1)	Thrombolytic
IL-4	Asthma
IL-4 Receptor	Asthma
IL-5	Allergy
IL-5 Receptor	Allergy
IgE	Allergic Asthma and Allergic Rhinitis
Eotaxin	Allergic Inflammatory Disease, Allergic Asthma
Eotaxin Receptor	Allergic Inflammatory Disease, Allergic Asthma
PDGF	Vascular Disease, Restinosis
PDGF beta Receptor	Vascular Disease, Restenosis
alpha.v.beta.3 Integrin	Inhibit Pathologic Bone Resorption

FIG. 8 B

Version Nov 5, 2003

Ontogeny of Proteolytic Immunity: IgM Serine Proteases *

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Running title: Proteolytic IgM antibodies

SUMMARY

We report the chemical activity of immunoglobulin μ and κ/λ expressed on the surface of B cells and secreted IgM antibodies (Abs) found in the preimmune repertoire. Most of the nucleophilic reactivity of B cells measured by formation of covalent adducts of a hapten amidino phosphonate diester was attributed to μ and κ/λ subunits of the B cell receptor. Secreted IgM Abs displayed superior nucleophilic reactivity than IgG Abs. IgM Abs catalyzed the cleavage of model peptide substrates at rates up to 344-fold greater than IgG Abs. Catalytic activities were observed in polyclonal IgM Abs from immunologically naïve mice and humans without immunological disease, as well as monoclonal IgM Abs to unrelated antigens. Comparison of several IgM Abs indicated divergent activity levels and substrate preferences, with the common requirement of a basic residue flanking the cleavage site. Fab fragments of a monoclonal IgM Ab expressed catalytic activity, confirming the V domain location of the catalytic site. The catalytic reaction was inhibited by the covalently reactive hapten probe and diisopropylfluorophosphate, suggesting a serine protease-like mechanism. These observations indicate the existence of serine protease-like BCRs and secreted IgM Abs as innate immunity components with potential roles in B cell development and Ab effector functions.

INTRODUCTION

Antigen-specific IgG Abs in autoimmune and alloimmune disease are described to catalyze chemical reactions (1-3). Examples of catalytic Abs raised by routine experimental immunization with ordinary antigens have also been published (4-7). However, no consensus has developed whether naturally occurring catalytic Abs represent rare accidents arising from adaptive sequence diversification processes or genuine enzymes with important functional roles. The major reason is that the turnover (k_{cat}) of antigen-specific IgG Abs is low. Some catalytic Abs express catalytic efficiencies (k_{cat}/K_m) comparable to conventional enzymes, but this is due to high affinity recognition of antigen ground state (reviewed in 8).

Certain enzymes cleave peptide bonds by a mechanism involving the formation of a transient covalent intermediate of the substrate and a nucleophilic residue present in the active site. The nucleophiles are generated by intramolecular activation mechanisms, *e.g.*, the activation of Ser/Thr side chain hydroxyl groups by hydrogen bonding to His residues, and can be detected by covalent binding to electrophilic phosphonate diesters (9,10). Using these compounds as covalently reactive analogs of antigens (CRAs), we observed that IgG Abs express nucleophilic reactivities comparable to trypsin (11). Despite their nucleophilic competence, IgG Abs display low efficiency proteolysis, presumably due to deficiencies in steps occurring after formation of the acyl-Ab intermediate, *viz.*, water attack on the intermediate and product release. Occupancy of the B cell receptor (BCR, surface Ig complexed to α and β subunits along with other signal transducing protein) by the antigen drives B cell clonal selection. Proteolysis by the BCR is compatible with clonal selection, therefore, only to the extent that the release of antigen fragments is slower than the rate of antigen-induced transmembrane signaling necessary for induction of cell division. Immunization with haptens mimicking the charge characteristics of the

transition state (12) has been suggested as a way to surmount the barrier to adaptive improvement of catalytic rate constants. Catalysis by 'designer' IgG Abs obtained by these means, however, also proceeds only slowly.

In mice and humans, the initial Ab repertoire consists of ~100 heritable VL and VH genes. Adaptive maturational processes expand the repertoire by several orders of magnitude. The initial BCR complex on the pre-B cell surface contains V-(D)-J rearranged Ig μ chains as a complex with surrogate L chains (reviewed in13). Precise assignment of the B cell differentiation stage at which cell division becomes antigen-dependent is somewhat ambiguous, but it is generally believed that non-covalent antigen binding to the pre-BCR is not required for initial cell growth. κ/λ chains replace the surrogate L chain at the later stages of antigen-driven B cell differentiation, which is accompanied by diversification *via* somatic hypermutation processes and continued gene rearrangements (14,15). V-(D)-J gene rearrangements allow development of specificity for individual antigens by IgM (16) but antigen binding affinities tend to be low compared to IgG Abs. Somatic mutations accumulating in the V domains following isotype switching to IgG promote high affinity antigen recognition. In some anatomic locations, IgM Abs can be extensively mutated and can display high affinity antigen binding (17). Loss of a membrane anchoring peptide at the C terminus of the H chain results in production of secreted IgM and IgG Abs.

Very little information is available about the developmental aspects of Ab catalysis. Here, we report the nucleophilic reactivity of secreted IgM and the Ig subunits expressed on the surface of B cells. Cell surface μ and κ/λ chains were the major sites of covalent reaction of a hapten CRA with B cells, and the magnitude of nucleophilic and proteolytic activities of secreted IgM Abs was consistently superior to IgG Abs.

EXPERIMENTAL PROCEDURES

Splenocyte-CRA binding. Synthesis of compounds **I-IV** and confirmation of their chemical identity have been published (11,18). Compounds **I, III** and **IV** are diphenyl phosphonate esters reactive with nucleophilic sites (9,10,18). Biotin incorporated in these compounds allowed the visualization of Ab-CRA adducts. Diisopropyl fluorophosphate (DFP) was from Sigma. BALB/c mice (5-6 weeks, female, Jackson Laboratories, Maine) were euthanized by cervical dislocation and splenocytes were prepared in RPMI-1640 (Gibco) by teasing apart the spleen and removing undissociated tissue (unit gravity sedimentation). Erythrocytes were lysed in hypotonic ammonium chloride (5 min; ACK Lysis Buffer, Cambrex, Walkersville, MD) and the cells washed twice with 10 mM sodium phosphate, pH 7.5, 137 mM NaCl, 2.7 mM KCl (PBS). B cells were isolated from splenocytes using a B cell negative selection isolation kit (Miltenyi, Auburn, CA) according to manufacturer's instructions and verified to be >95% CD19+ by flow cytometry as described below. Viability was determined using 0.05% Trypan Blue (90-95%). The cells (2-5 x 10^6 cells) were incubated with hapten CRA **I** or compound **II** (37°C; final DMSO concentration 1%) in 0.5 ml PBS, washed thrice and treated with 100 µl anti-CD16/32 Ab (10 µg/ml µl; BD Pharmingen, San Diego, CA; 5 min, 4°C) to block Ab binding to Fc receptors. Staining was with FITC-conjugated streptavidin (1 µg/ml or as stated; Molecular Probes, Eugene, Oregon) and PE-conjugated rat monoclonal Ab to CD19 (10 µg/ml; Caltag, Burlingame, CA) in 100 µl for 20 min at 4°C. Following further washing with PBS (2x), the cells were fixed with 2% paraformaldehyde (1 hour, 4°C), washed once and resuspended in PBS. In control incubations, an equivalent concentration of PE conjugated isotype-matched rat Ab to an irrelevant antigen (Pharmingen) replaced the anti-CD19 Ab. Deconvolution microscopy was performed employing an Olympus IX-70 inverted microscope and Applied Precision Delta work

station (SoftWoRx™ software; ref 19). Stained cells were subjected to multiple acquisitions at a thickness of 0.25 μm, and the images were stacked. The images were subjected to deconvolution (5 iterations) for each probe (FITC; λex 488nm, λem 525nm; DAPI; λex 350nm, λem 470nm; phycoerythrin; λex 565nm, λem 578nm). Flow cytometry was performed in the Baylor Medical College Core Facility (EPICS XL-MCLs Beckman-Coulter flow cytometer, EXPO32 software). Instrument calibration to minimize cross-detection of PE and FITC was done using cells stained individually with these fluorochromes. Forward and side scatter measurements allowed exclusion of dead cells from the gated cell population. CRA-stainable cells were identified as the population showing staining above the level observed for compound **II** staining. CRA stainable CD19+ cells were estimated by subtraction of background observed using the isotype-matched Ab. Cell extraction was by treatment with the detergent CHAPS (12 mM, 2 hours at 4°C). The extract was centrifuged (10,000 g, 30 min), the supernate diluted with PBS to 1 mM CHAPS and then subjected to affinity chromatography using goat polyclonal Abs (IgG) to mouse μ, γ, δ, λ and κ chains (Caltag) immobilized on Protein G-Sepharose columns (100 μl settled gel; 0.6 x 5cm columns; Pharmacia, Piscataway, NJ). For this purpose, the Abs (50 μg) were mixed with the Protein G gel in a column (15 min, 4°C) in PBS containing 1 mM CHAPS (PBS-CHAPS), the gel allowed to settle, the unbound fraction collected and the columns washed with PBS-CHAPS. The cell extract (1.4 ml; diluted to 1 mM CHAPS; from $3x10^6$ cells) was passed through the column, the column washed with PBS-CHAPS (9 volumes) and bound proteins were eluted with 100 mM glycine-HCl, pH 2.7 (8 column volumes) and subjected to reducing SDS-polyacrylamide gel electrophoresis (4-20%, Bio-Rad). Protein-CRA adducts were visualized by staining nitrocellulose electroblots of the gels with streptavidin-peroxidase as in (11). For immunoblotting, the blots were stained with goat polyclonal Abs (IgG) to mouse μ, γ, δ, λ and κ

chains followed by peroxidase conjugated rabbit anti-goat IgG (Fc specific, 1:1000; Pierce) as in (11). Nominal mass values were computed by comparison with standard proteins (14 kD –220 kD; Pharmacia).

Secreted Ab-CRA binding. Human serum Abs were from subjects without evidence of infection or immunological disease (2 females, 3 males; age 23-45 y). Murine serum Abs were from BALB/c mice (purchased from Harlan, Indianapolis, IN; pooled from 150 mice; 8-12 weeks). Murine monoclonal IgM Abs used here are directed against major histocompatibility antigens (clones corresponding to catalog #8702, 8704, 9008, 9010, 9020; cell-free ascites; Cedarlane, Ontario, Canada). Monoclonal IgM Yvo is from a patient with Waldenstrom's macroglobulinemia (20). All monoclonal IgM Abs contain κ chains. The 4 murine monoclonal IgG Abs used here were: clone c23.4 (anti-VIP; ref 6), clone c39.1 (anti-glucagon; S. Paul and coworkers, unpublished); ATCC clones HP6045 (anti-Fab_2, γ) and ATCC clone HP6054 (anti- Ig λ chain). All monoclonal IgG Abs contain γ2a heavy chains and κ light chains. Serum or ascites fluid (1ml) was mixed for 1h with 1 ml Sepharose 4B conjugated rat anti-mouse IgM Abs (settled gel; Zymed, San Francisco, CA) or agarose conjugated goat anti-human IgM Abs (Sigma, St. Louis, MO) with IgM binding capacities 0.8 and 3 mg, respectively, in 50 mM Tris-HCl, pH 7.5, 0.1 mM CHAPS (buffer A). The unbound fraction was recovered and the gel washed with 20 buffer A volumes taking care that protein in the effluent had returned to undetectable levels prior to elution ($A_{280} < 0.001$). Elution was with 100 mM glycine pH 2.7 (0.5 ml/fraction into 25 µl 1M Tris-HCl, pH 9.0). Further purification was on a Superose-6 FPLC gel filtration column (1x30 cm; 0.25 ml/min; Pharmacia) in two different solvents: 50 mM Tris-HCl, pH 7.7, 0.1 M glycine, 0.15 M NaCl, 0.025% Tween-20 (buffer B) or 6 M guanidine hydrochloride in buffer B adjusted to pH 6.5 with HCl (buffer C). Prior to column fractionation,

the affinity purified IgM was dialyzed against buffer C. Column calibration was with thyroglobulin (660 kD), IgG (150 kD) and albumin (67 kD). IgM with Mr 900 kD eluted close to the void volume of the column. IgM was renatured following buffer C chromatography by dialysis against buffer B (21). IgM Yvo, a cryoglobulin, was purified from serum by repetitive warming (37°C) and cooling (4°C; 3 cycles; ref 20) followed by affinity chromatography on the anti-human IgM column. IgG was purified on Protein G-Sepharose columns (21) using as starting material the unbound fraction from the anti-IgM columns or cell-free ascites. FPLC gel filtration of IgG was as described for IgM except that a Superose 12 column was employed. Fab fragments were prepared by digesting IgM (300 µl, 1 mg/ml) with agarose conjugated pepsin (0.6 ml gel, 30 min, 37°C) in 100 mM sodium acetate, pH 4.5, 150 mM NaCl, 0.05% NaN_3, 0.1 mM CHAPS) as recommended by the manufacturer (Pierce). The unbound fraction was dialyzed against buffer B, purified by FPLC gel filtration on a Superose 12 column and dialyzed against 50 mM Tris-HCl, pH 7.7, 0.1 M glycine, 0.1 mM CHAPS. Total protein was determined by the bicinchoninic acid method (Pierce). Immunoblotting of SDS-gels contanining murine Abs was as in the preceding section. Human Ab gels were immunoblotted using peroxidase conjugated goat anti-human µ, anti-human κ and anti-human λ Abs (Sigma, St Louis, MO).

Purified Abs were treated with the biotinylated CRAs in 50 mM Tris, HCl, 100 mM glycine, 0.1 mM CHAPS, pH 7.7 at 37°C. Formation of Ab-CRA adducts was determined by SDS-electrophoresis as in the preceding section. Band intensities are expressed in arbitrary area units (AAU) determined by densitometry (11). Initial velocities were computed as the slopes of progress curves plotted as a function of time (initial 60 min).

Proteolysis assays. Cleavage of the amide bond linking aminomethylcoumarin to the C terminal

amino acid in peptide-AMC substrates (Peptide International, Louisville, KY or Bachem, King of Prussia, PA) was measured in 50 mM Tris HCl, pH 7.7, 0.1 M glycine, 0.025% Tween-20 at 37°C in 96-well plates by fluorimetry (λ_{ex} 360 nm, λ_{em} 470 nm; Varian Cary Eclipse) (21). Authentic aminomethylcoumarin was used to construct a standard curve. Kinetic parameters were obtained by fitting rate data obtained at increasing concentrations of peptide-AMC substrates to the Michaelis-Menten-Henri equation: $v=(V_{max}[S])/(K_m+[S])$. Progress curves in the presence of inhibitors were fitted to the equation: $[AMC]/[AMC]_{max}=1-e^{-k_{obs}\cdot t}$. where $[AMC]_{max}$ is the AMC concentration in the absence of inhibitor. IC50 (concentration yielding 50% inhibition) was obtained from the equation: % inhibition = $100/(1+10^{\log IC50-\log[Inhibitor]})$ with the curve forced through 0.

RESULTS

Irreversible CRA-B cell binding. Hapten CRAs such as compound **I** (Fig 1) react irreversibly with nucleophilic sites in conventional serine proteases and Abs (9-11,18). To evaluate the nucleophilic reactivity expressed on the surface of B cells in the preimmune repertoire (*viz.*, the repertoire developed spontaneously without purposeful immunological challenge), viable splenocytes from BALB/c mice were treated with hapten CRA **I**. The control compound **II** is identical in structure to hapten CRA **I**, except that the phosphonate group is not esterified, which results in loss of covalent reactivity with nucleophilic residues (11,18). Treatment with hapten CRA **I** resulted in staining of most of cells at levels greater than compound **II**, with a minority of the cells displaying intense staining (11±2, N 3 experiments; determined by counting 400 lymphocytes using a UV microscope). All of the CRA **I**-stained cells displayed lymphocytic

morphology, with no evident staining of monocytes or the occasional basophil. No loss of viability of the cells was evident following incubation with CRA I or compound II, as determined by trypan blue exclusion. Flow cytometry confirmed the microscopy results. Seventy nine percent of the CRA I-treated cells displayed fluorescence intensities exceeding the compound II-treated cells, including a minority subpopulation with very high fluorescence intensity (14 %; subpopulation 2 in Fig 2A). In 3 repeat experiments, the proportion of CRA I-stained cells that were positive for the B cell marker CD19 was 82±4% (Fig 2B). Deconvolution microscopy indicated that the fluorescence pattern due to hapten CRA I binding was nearly coincident with the anti-CD19 Ab fluorescence pattern (Fig 2C-E). Most of the CRA fluorescence was restricted to the surfaces of the B cells (Fig 2F).

To identify the nucleophilic molecules on the cell surface, purified B cells were labeled with CRA I, detergent extracts of the cells were boiled and then analyzed by SDS-electrophoresis. Only limited CRA-containing proteins were evident (Fig 3A). As expected, silver staining revealed the presence of heterogeneous species, reflecting the complex protein constitution of the cells. The mass of the predominant CRA adduct band was 70 kD, and this band was stainable by anti-μ chain Ab (Fig 3B). Smaller amounts of CRA-containing bands were evident at 25 kD, 40 kD, 50 kD, 55-60 kD, 90-135 kD and 140 kD. The bands at 55-60 kD and 140 kD were stainable by the anti-μ Ab, and the bands at 25 kD and 50 kD were stainable with anti- κ/λ Ab. The anomalous μ and κ/λ bands at mass range different from the full-length monomer proteins presumably represent unreduced oligomers, breakdown products and truncated B cell Ig products, as also observed in previous studies of secreted Abs and B cell extracts (22-24). The minor bands at 40 kD and 90-135 kD that were not stainable with Abs to μ, γ, κ/γ (Fig 3 B) and δ chains (not shown) presumably represent non-Ig proteins. No CRA-containing adduct

corresponding to Ig γ chains were detected. Immunoblotting of the cell extracts identified a band at 50 kD stainable with anti-γ Ab, but the band was visible only in highly overexposed gels, suggesting that only small amounts of γ chains were present in the extract.

Confirmation that the CRA I adducts contain Ig subunits was by affinity chromatography on columns of immobilized Abs to μ, δ, γ and κ/λ chains followed by SDS-electrophoresis (Fig 3C). CRA-containing μ and κ/λ bands were evident in eluates from the anti-μ and anti-κ/λ columns. Recovery of CRA-containing μ chains in the eluate from the anti-κ/λ column can be explained by the presence of disulfide bonded light and heavy chain complexes on the cell surface. No CRA-containing bands were evident in eluates from the anti-γ and anti-δ columns (not shown), but this can not be interpreted to reflect deficient γ/δ chain nucleophilic reactivity, as these proteins are expressed only at low levels in B cells from immunologically naïve mice. To determine the proportion of overall cellular CRA staining attributable to complexation with Ig subunits, the B cell extract was fractionated on a single affinity column composed of immobilized Abs to μ and κ/λ chains. Eighty percent of the total CRA content of the cells was adsorbed by the column (not shown), determined by densitometry of the biotin-containing bands in the unbound fraction and the extract loaded on the column. Taken together, these observations indicate that most of the CRA staining of intact B cells is attributable to irreversible binding to surface Ig, with the μ chain accounting for most of the covalent reactivity.

Nucleophilic reactivity of secreted IgM. The initial velocity for formation of hapten CRA I adducts by IgM purified from the pooled serum of immunologically naïve BALB/c mice was 40-fold greater than by IgG (Fig 4A; values are sums of velocities for the reactions occurring at the two Ab subunits expressed per unit concentration of intact Abs). The velocity difference is 8-fold when expressed per unit combining site concentration^2 (10 and 2 combining sites, respectively,

in IgM and IgG). Three CRA-containing bands were observed in reducing SDS-gels of the IgM reaction mixtures at 70, 50 and 25 kDa (Fig 4B). The 70 kDa and 25 kDa bands were stainable with anti-μ and anti-κ/λ Abs, respectively. The 50 kDa band was stainable with anti-μ Ab and presumably represents a μ breakdown product. Two CRA-containing bands corresponding to γ and κ/λ chains were observed in reducing gels of the IgG reaction mixtures. Similar results were obtained with a panel of 6 randomly selected monoclonal IgM Abs (5 murine and 1 human) and 4 monoclonal IgG Abs (all murine). The monoclonal IgM Abs uniformly displayed superior rates of irreversible CRA I binding compared to the IgG Abs (Fig 4C; mean ± SEM: $62.6 \pm 24.4 \times 10^4$ and $1.9 \pm 0.4 \times 10^4$ AAU/μM Ab/hour, respectively; $P<0.01$, Mann-Whitney U test, 2 tailed). Consistent with the polyclonal Ab experiments, the μ chain accounted for most of the covalent binding in the polyclonal and monoclonal IgMs, but smaller levels of binding at the κ/λ chain subunit were also observed for every Ab preparation (for clarity, μ chain and the corresponding κ/λ chain data points from individual IgM preparations are connected in Fig 4C; data are expressed per μM subunit concentration to allow ready comparison). The 4 monoclonal IgG Abs contain γ2a heavy chains, and all monoclonal IgM/IgG Abs contain κ light chains. No attempt was made to determine the nucleophilic reactivity of various γ chain isotypes. However, the polyclonal Ab data indicate that the average nucleophilic reactivity of the IgG isotype mixture in blood is lower than the IgM reactivity. A similar argument can be presented in regard to antigenic specificity. The 5 murine IgM Abs and 4 IgG Abs were raised by experimental immunization and bind different antigens (MHC antigens, VIP, glucagon, Ig subunits; refs 6,25 and specifications provided by the manufacturers).The sixth monoclonal IgM was from a patient with Waldenström's macroglobulinemia with unknown antigenic specificity (20). The monoclonal IgM Abs uniformly displayed superior reactivity to IgG Abs, suggesting that

divergent antigenic specificities do not account for the reactivity difference.

One of the monoclonal IgM Abs, Yvo, was employed to help define the structural requirements favoring hapten CRA covalent binding. Compound **II**, which contains the unesterified phosphonate, did not form adducts with the IgM at incubation times up to 3 hours (reaction conditions as in Fig 3B). Similarly, the neutral hapten CRA **III** devoid of the amidino group and the hapten CRA **IV** with weak leaving groups (methyl instead of phenyl groups) failed to form detectable adducts with this IgM Ab. These reactivity characteristics are similar to those of IgG Abs reported previously (11).

Secreted IgM catalytic activity. The catalytic activity of polyclonal IgM and IgG prepared from pooled mouse serum was initially measured using Glu-Ala-Arg-AMC as substrate (Fig 5A). Cleavage of the amide bond linking the AMC to the C terminal Arg residue of this peptide has been validated as a surrogate for peptide bond hydrolysis by IgG Abs (21). Cleavage of Glu-Ala-Arg-AMC by polyclonal murine and human IgM fractions proceeded at rates 344-fold and 237-fold greater, respectively than the IgG fractions from the same sera (computed from initial velocity data; expressed per unit intact Ab concentration). If all 10 IgM valencies[2] and both IgG valencies are filled, the velocities for individual combining sites of murine and human IgM are 69-fold and 47-fold greater than the corresponding IgG velocities. Consistent with the irreversible binding data in the preceding section, Glu-Ala-Arg-AMC cleavage by murine polyclonal IgM was inhibited by hapten CRA **I** (Fig 5B) and the serine protease inhibitor diisopropylfluorophosphate (not shown; 63% and 93% inhibition at 30 μM and 100 μM DFP, respectively after 12 hours). The deviation of the progress curve from linearity in the presence of CRA **I** suggests an irreversible inhibition mode (26). Progressively increasing inhibition of the murine IgM activity (9-100%) at increasing hapten CRA **I** concentrations (10-300 μM) was

evident (IC50 42 μM; not shown). Similar results were obtained using human polyclonal IgM as the catalysts (IC50 value for hapten CRA I inhibition, 111 μM).

Contamination of IgM with conventional proteases was studied by methods employed previously to validate IgG and Ab light chain enzymatic activities (21,27). The IgM obtained by affinity chromatography on the anti-μ column displayed essentially identical levels of catalytic activity as the 900 kD IgM fraction obtained by further purification by FPLC gel filtration (Fig 6A). This fulfils the criterion of purification to constant specific activity required for assignment of enzymatic activity to IgM. Next, we examined IgM treated with 6M guanidine hydrochloride to dissociate any noncovalently associated contaminants. For this purpose, the affinity purified IgM was subjected to three cycles of gel filtration in 6M guanidine hydrochloride (Fig 6B) and the 900 kD fraction from the final gel filtration cycle was renatured by dialysis. Time-dependent Glu-Ala-Arg-AMC cleavage by IgM subjected to these procedures was observed (Fig 6C).

Substrate selectivity of the polyclonal IgM preparations and 6 monoclonal IgM Abs was studied using a panel of 10 peptides-AMC conjugates. The rates shown in Table 1 were computed as slopes of the progress curves. Only substrates containing a basic residue at the cleavage site were hydrolyzed by the IgM Abs. No hydrolysis was detected with substrates containing acidic and neutral residues at the cleavage site. All 6 monoclonal IgM Abs displayed catalytic activity, but the activity levels for different Abs were not identical (varying, for example, over a 24-fold range with Glu-Ala-Arg-AMC as substrate). The Abs displayed different substrate selectivity profiles. For example, the ratio of Glu-Ala-Arg-AMC and Ile-Glu-Gly-Arg-AMC cleavage rates varied from 0.9 to 30.0 for the 5 murine monoclonal IgMs, and the human monoclonal IgM cleaved the former substrate at a robust rate without cleaving the latter substrate detectably (Fig 7). Hydrolysis of Gly-Gly-Arg-AMC and Gly-Gly-Leu-AMC by IgM 9020 was compared to

confirm the requirement for a basic residue at the cleavage site. These substrates are identical except for the Arg-AMC/Leu-AMC linkage, eliminating the possibility of confounding remote residue effects. Cleavage of Gly-Gly-Arg-AMC was detectable, but cleavage of Gly-Gly-Leu-AMC was not (12.6 ± 0.6 and < 0.13 μM AMC/μM Ab/hour, respectively).

The constant domain scaffold in the 5 murine monoclonal IgM Abs is identical. Observations of divergent catalytic activity levels and substrate selectivities suggested that the catalytic site is located in the V domains. To confirm this, IgM Yvo was digested with immobilized pepsin and Fab fragments were purified by gel filtration as the 55 kD protein peak (Fig 8A). Concentration dependent cleavage of Glu-Ala-Arg-AMC by the Fab fragment was observed (Fig 8B). Next, we considered the possibility that pepsin released from the column could be responsible for the observed Fab activity. The pH optimum of pepsin is 1.5-2.7 depending on the substrate (28). The catalysis assays were repeated in 0.1 M glycine, pH 2.7, 1 mM CHAPS. At Fab concentrations affording readily detectable catalytic activity at neutral pH (Fig 8B), no detectable cleavage of Glu-Ala-Arg-AMC by the Fab was evident at pH 2.7. The cleavage site preference of pepsin (hydrolysis on the C terminal side of aromatic and hydrophobic residues) is dissimilar to the basic residue preference of IgM Abs. Purified pepsin did not cleave Glu-Ala-Arg-AMC under conditions yielding readily detectable catalysis by the Fab (675 nM pepsin; other reaction conditions as in Fig 8B). These data indicate that pepsin contamination is not a factor in the observed Fab activity.

Determination of reaction rates for 4 IgM preparations at increasing Glu-Ala-Arg-AMC concentrations indicated typical enzymatic kinetics (polyclonal murine and human IgM, monoclonal IgM 9020 and IgM 9008). The rates were saturable at excess substrate concentration and consistent with the Michaelis-Menten-Henri kinetics (Table 2). Observed K_m values were in

the high micromolar range, reminiscent of the recognition characteristics of conventional proteases. Catalytic antibodies that are adaptively specialized to recognize individual antigens, on the other hand, display K_m values in nanomolar to low micromolar range, e.g., IgG c23.5 shown in Table 2 cleaves VIP with K_m 0.4 nM (from ref 6). Apparent k_{cat} values for the IgMs exceed those reported previously for IgG (21).

DISCUSSION

IgM Abs, the first class of Abs produced by B cells, displayed superior nucleophilic and catalytic reactivities compared to IgG Abs. This contrasts with the noncovalent antigen binding function of Abs, which improves adaptively over the course of the immune response. The nucleophilic and catalytic IgM activities were identified in unimmunized mice and healthy humans. Preferential IgM recognition of the positive charged group adjacent to the phosphonate group of the hapten CRAs and the scissile bond in peptide substrates presumably reflect an intrinsic property of the preimmune Ab repertoire. This noncovalent recognition motif enables low affinity interactions with the peptide substrates, whereas traditional noncovalent epitope-paratope binding is characterized by high affinity antigen recognition (nanomolar range K_d).

Hapten CRA I was validated previously as a probe for nucleophilic reactivities expressed by serine proteases, including IgG Abs (11,28). The extent of irreversible CRA binding activity correlates approximately with the catalytic activity (11,29). In the present study, hapten CRA I adducts were located in close proximity to CD19 on the surface of B cells. The latter protein fulfills a signal transducing role as a component of the BCR throughout B cell development (30). Immunochemical and affinity chromatography studies suggested that the majority of the B cell

surface staining is attributable to covalent binding by Ig subunits, with the μ chain providing the dominant contribution, and κ/λ chains, a smaller contribution. This is consistent with the superior nucleophilic reactivity of the μ chain subunit of secreted IgMs. The control phosphonic acid hapten, which stained the cells poorly, does not react with nucleophiles due to the poor electrophilicity of the phosphorus atom (11,18). Monoclonal BCRs were not included here, but all six monoclonal IgM Abs examined expressed nucleophilic reactivity, suggesting that the reactivity may also be expressed by a significant proportion of BCRs. A minority of the B cells was stained intensely by the CRA. These cells are of interest as a potential source of catalysts in future studies. Observations that both Ig subunits express nucleophiles are consistent with the ability of light and heavy chains to independently catalyze the cleavage of peptide bonds in the absence of their partner subunit (31). Site-directed mutagenesis studies have indicated a serine protease-like catalytic triad in the light chain of an IgG Ab (32) and the heavy chain of other IgG Abs is reported to contain nucleophilic Ser residues (*e.g.*, 33).

Functional roles for serine protease activities have been deduced in B cell developmental processes, but the molecules responsible for the activities have not been identified to our knowledge. The serine protease inhibitors DFP and α-1 antitrypsin inhibit mitogen induced B cell division (34,35) and up-regulate the synthesis of certain Ab isotypes by cultured B cells (35). The DFP-sensitive enzyme is B cell-associated and prefers Arg-containing substrates (36). Serine protease inhibitors are reported to inhibit anti-IgM induced BCR signal transduction (37), and anti-IgM mediated B cell activation is correlated with the appearance of a serine protease activity on the cell surface (38). Undoubtedly, conventional serine proteases may contribute to B cell regulation, but it remains that the major CRA binding components on the B cell surface evident in the present study are the BCRs themselves. It is logical to hypothesize, therefore, that

stimulation of BCR nucleophilic sites may influence B cell development. Such compounds include naturally occurring serine protease inhibitors and reactive carbonyl compounds capable of irreversible binding to nucleophilic amino acids (39).

Observations of divergent levels of catalytic activity of monoclonal IgM Abs, their differing substrate preference and retention of the activity in the Fab fragments suggest that the catalytic site is located in the V domains. The superior activity of IgM compared to IgG cannot be ascribed to avidity effects, as the catalysis assays are conducted in solution phase and at excess concentrations of the small peptide substrate. These conditions will not support binding of a single peptide molecule by more than one Ab valency. The following explanations can be presented for superior catalysis by IgM Abs. First, loss of catalytic activity may be attendant to V domain somatic diversification after isotype switching from IgM to IgG. Second, distinctive IgM constant domain characteristics may be important in maintaining the integrity of the catalytic site, in which case isotype switching itself may result in reduced catalytic activity. These explanations are not mutually exclusive. Both explanations are consistent with the argument that catalysis is a disfavored phenomenon in the advanced stages of B cell development (as efficient BCR catalysis will result in reduced BCR occupancy). We did not attempt to address these points experimentally in the present study. However, the monovalent Fab studies suggested that disruption of the constant domain architecture of IgM is deleterious for catalysis. The Fab preparations displayed ~10-fold lower activity than computed for the individual combining sites of pentameric IgM. Pepsin employed to prepare Fab cleaves μ chains on the C terminal side of the CH2 domain (40), which is distinguished by its conformational flexibility (41). Alterations of antigen binding activity when the same V domains are expressed as full-length IgG Abs belonging to different isotypes are described (*e.g.*, 42), but we are not aware of IgM-IgG V

domain swapping experiments in the literature. Positive cooperativity effects such as are described for antigen binding by the two IgG combining sites (43), could theoretically furnish favorable contributions in catalysis. The temporal sequence of events as individual IgM combining sites bind antigen has not been elucidated, but the hypothesis of positive cooperativity is not supported by findings that only 5 of the 10 IgM combining sites are filled at excess antigen concentration (*e.g.*, 44).

Our screening experiments were restricted to a few IgMs and a few commercially available substrates. Additional studies are necessary to define the physiological substrates for IgM Abs and the upper limit for catalytic rates. However, certain conclusions can be reached from the available data. Apparent turnover numbers (k_{cat}) for the IgM preparations were as high as 2.8/min. Serum IgM concentrations (1.5-2.0 mg/ml; ~2 μM) are ~ 3-4 orders of magnitude greater than conventional enzymes (for example, thrombin found at ng - μg/ml in serum as a complex with antithrombin III; ref 45), and IgM k_{cat} values are ~2 orders of magnitude smaller than conventional serine proteases. If catalysis proceeds at the rate observed *in vitro*, 2 μM human IgM with turnover 2.8/min will cleave ~24,000 μM antigen present at excess concentration ($>>K_m$) over 3 days (corresponding to the approximate half-life of IgM in blood). Maximal velocity conditions can be approached in the case of antigens present at high concentrations, *e.g.*, albumin and IgG in blood; polypeptides accumulating at locations close to their synthetic site, such as thyroglobulin in the lumen of thyroid follicles; and bacterial and viral antigens in heavily infected locations. Inhibitors regulate the activity of conventional proteases *in vivo* such as the enzymes responsible for blood coagulation. Unregulated catalysis may lead to disruption of homeostasis. Inhibitory mechanisms regulating conventional enzymes are conceivable in regard to IgM proteolysis.

Identification of promiscuous IgM proteolytic activities in the preimmune repertoire raises important question concerning the existence antigen-specific catalytic IgM Abs. Under conditions of limiting antigen concentration, catalyst competence is measured as the k_{cat}/K_m parameter ($K_m \approx K_d^3$). As illustrated for the anti-VIP IgG in Table 2, large gains in catalytic competence occur due to enhanced antigen binding affinity (reduced K_m). Certain polypeptides are recognized by IgM Abs present in the preimmune repertoire with high affinity, for example, the superantigens Staphylococcal Protein A and HIV gp120[4] are recognized by IgM Abs containing VH3 family domains with K_d in the nanomolar range (46,47). Moreover, specific IgM Abs with improved affinity for individual antigens emerge by adaptive V domain maturation processes (16,48). Similarly, future study of catalytic IgMs specialized to recognize individual autoantigens is of interest. IgM Abs from patients with autoimmune disease express glycosidase activity (49). Autoimmune humans and mice tend to synthesize catalytic Abs at increased levels (50-53), and a proteolytic IgG preparation to VIP is shown to interfere with the physiological smooth muscle relaxant effect of VIP (54).

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REFERENCES

1. Paul, S., Volle, D. J., Beach, C. M., Johnson, D. R., Powell, M. J., and Massey, R.J. (1989) *Science* **244**, 1158-1162

2. Shuster, A. M., Gololobov, G. V., Kvashuk, O. A., Bogomolova, A. E., Smirnov, I. V., and Gabibov, A. G. (1992) *Science* **256**, 665-667

3. Lacroix-Desmazes, S., Moreau, A., Sooryanarayana, B. C., Stieltjes, N., Pashov, A., Sultan, Y., Hoebeke, J., Kazatchkine, M. D., and Kaveri, S. V. (1999) *Nat. Med.* **5**, 1044-1047

4. Raso, V., and Stollar, B. D. (1975) *Biochemistry* **14**, 591-599

5. Kohen, F., Kim, J. B., Linder, H. R., Eshhar, Z., and Green B. (1980) *FEBS Lett.* **111**, 427-431

6. Paul, S., Sun, M., Mody, R., Tewary, H. K., Mehrotra, S., Gianferrara, T., Meldal, M., and Tramontano, A. (1992) *J. Biol. Chem.* **267**, 13142-13145

7. Hifumi, E., Okamoto, Y., and Uda, T. (1999) *J. Biosci. Bioengin.* **88**, 323-327

8. Paul, S., Ed. (2000) *Chemical Immunology: Catalytic Antibodies,* Vol. 77, pp. 1-161, S. Karger GmbH, Basel, Switzerland

9. Oleksyszyn, J., and Powers, J. C. (1994) in *Methods in Enzymology,* (Barrett, A. J., ed) Vol. 244, pp. 423–441, Academic Press, San Diego, CA

10. Sampson, N. S., and Barton, P. A. (1991) *Biochemistry* **30**, 22255-22263

11. Planque, S., Taguchi, H., Burr, G., Bhatia, G., Karle, S., Zhou, Y.-X., Nishiyama, Y., and Paul, S. (2003) *J. Biol. Chem.* 278, 20436-20443

12. Tramontano, A., Janda, K. D., and Lerner, R. A. (1986) *Science* 234, 1366–1570

13. Melchers, F., ten Boekel, E., Seidl, T., Kong, X. C., Yamagami, T., Onishi, K., Shimizu, T., Rolink, A. G., and Andersson, J. (2000) *Immunol. Rev.* 175, 33-46

14. Prak, E. L., and Weigert, M. (1995) *J. Exp. Med.* 182, 541-548

15. Papavasiliou, F., Casellas, R., Suh, H., Qin, X. F., Besmer, E., Pelanda, R., Nemazee, D., Rajewsky, K., and Nussenzweig, M. C. (1997) *Science* 278, 298-301

16. Xu, J. L., and Davis, M. M. (2000) *Immunity* 13, 37-45

17. Dunn-Walters, D. K., Hackett, M., Boursier, L., Ciclitira, P. J., Morgan, P., Challacombe, S. J., and Spencer, J. (2000) *J. Immunol.* 164, 1595-1601

18. Nishiyama, Y., Taguchi, H., Luo, J., Zhou, Y.-Z., Burr, G., Karle, S., and Paul, S. (2002) *Arch. Biochem. Biophys.* 402, 281–288

19. Poindexter, B. J., Pereira-Smith, O., Wadhwa, R., Buja, L. M., and Bick, R. J. (2002) *Microscopy & Analysis* 89, 21-23

20. Shaw, D. C., Shultz, B. B., Ramsland, P. A. and Edmundson, A. B. (2002) *J. Mol. Recog.* 15, 341-348

21. Kalaga, R., Li, L., O'Dell, J., and Paul, S. (1995) *J. Immunol.* 155, 2695-2702

22. Marks, R., and Bosma, M. J. (1985) *J. Exp. Med.* 162, 1862-1877

23. Li, L., Sun, M., Gao, Q. S., and Paul, S. (1996) *Mol. Immunol.* **33**, 593-600

24. Malynn, B. A., Shaw, A. C., Young, F., Stewart, V., and Alt, F. W. (2002) *Mol. Immunol.* **38**, 547-556

25. Ozato, K., Mayer, N. M., and Sachs, D. H. (1982) *Transplantation* **34**, 113-120

26. Marangoni, A. G. (2003) *Enzyme Kinetics: A Modern Approach,* pp. 70-79, John Wiley and Sons, Hoboken, NJ

27. Paul, S., Li, L., Kalaga, R., Wilkins-Stevens, P., Stevens, F. J., and Solomon, A. (1995) *J. Biol. Chem.* **270**, 15257-15261

28. Cornish-Bowden, A., and Knowles, J. (1969) *Biochem. J.* **113**, 353-362

29. Paul, S., Tramontano, A., Gololobov, G., Zhou, Y.-X, Taguchi, H., Karle, S., Nishiyama, Y., Planque, S., and George, S. (2001) *J. Biol. Chem.* **276**, 28314-28320

30. Sato, S., Ono, N., Steeber, D. A., Pisetsky, D. S., and Tedder, T. F. (1996) *J. Immunol.* **157**, 4371-4378

31. Gao, Q.-S., Sun, M., Rees, A., and Paul, S. (1995) Site-directed mutagenesis of proteolytic antibody light chain. *J. Mol. Biol.* **253**, 658-664

32. Hatiuchi, K., Hifumi, E., Mitsuda, Y., and Uda, T. (2003) *Immunol. Lett.* **86**, 249-257

33. Kolesnikov, A. V., Kozyr, A. V., Alexandrova, E. S., Koralewski, F., Demin, A. V., Titov, M. I., Avalle, B., Tramontano, A., Paul, S., Thomas, D., Gabibov, A. G., and Friboulet, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 13526-13531

34. Ku, G. S., Quigley, J. P., and Sultzer, B. M. (1981) *J. Immunol.* **126**, 2209

35. Jeannin, P., Lecoanet-Henchoz, S., Delneste, Y., Gauchat, J. F., and Bonnefoy, J. Y. (1998) *Eur. J. Immunol.* **28**, 1815-1822

36. Ku, G. S., Quigley, J. P., and Sultzer, B. M. (1983) *J. Immunol.* **131**, 2494-2499

37. Mizuguchi, J., Utsunomiya, N., Nakanishi, M., Arata, Y., and Fukazawa, H. (1989) *Biochem. J.* **263**, 641-646

38. Biro, A., Sarmay, G., Rozsnyay, Z., Klein, E., and Gergely, J. (1992) *Eur. J. Immunol.* **22**, 2547-2553

39. Crabb, J. W., O'Neil, J., Miyagi, M., West, K., and Hoff, H. F. (2002) *Protein Sci.* **11**, 831-840

40. MacKenzie, M. R., Gutman, G. A., and Warner, N. L. (1978) *Scand. J. Immunol.* **7**, 367-370

41. Roux, K. H., Strelets, L., Brekke, O. H., Sandlie, I., Michaelsen, T. E. (1998) *J. Immunol.* **161**, 4083-4090

42. Morelock, M. M., Rothlein, R., Bright, S. M., Robinson, M. K., Graham, E. T., Sabo, J. P., Owens, R., King, D. J., Norris, S. H., Scher, D. S., Wright, J. L., and Adair, J. R. (1994) *J. Biol. Chem.* **269**, 13048-13055

43. van Erp, R., Gribnau, T. C., van Sommeren, A. P., and Bloemers, H.P. (1991) *J. Immunol. Methods* **140**, 235-241

44. Chavin, S. I., and Franklin, E. C. (1969) *J. Biol. Chem.* **244**, 1345-1352

45. Chen, T. Y., Huang, C. C., and Tsao, C. J. (1993) *Am. J. Hematol.* **44**, 276-279

46. Rohen, P. W., Salem, A. N., and Silverman, G. J. (1995) *J. Immunol.* **154**, 6437-6445

47. Berberian, L., Goodglick, L., Kipps, T. J., and Braun, J. (1998) *Science* **261**, 1588-1591

48. Ballard, D. W., Kranz, D. M., Voss, E. W., Jr. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 5071-5074

49. Saveliev, A. N., Ivanen, D. R., Kulminskaya, A. A., Ershova, N. A., Kanyshkova, T. G., Buneva, V. N., Mogelnitskii, A. S., Doronin, B. M., Favorova, O. O., Nevinsky, G. A., and Neustroev, K. N. (2003) *Immunol. Lett.* **86**, 291-297

50. Tawfik, D., Chap, R., Green, B., Sela, M., and Eshhar, Z. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2145-2149

51. Li, L., Kaveri, S., Tyutyulkova, S., Kazatchkine, M., and Paul, S. (1995) *J. Immunol.* **154**, 3328-3332

52. Matsuura, K., Ikoma, S., Sugiyama, M., Funauchi, M., and Sinohara, H. (1998) *Immunol.* **95**, 26-30

53. Bangale, Y., Karle, S., Zhou, Y.-X., Lan, L., Kalaga, R., and Paul, S. (2003) *FASEB J.* **17**, 628-635

54. Berisha, H. I., Bratut, M., Bangale, Y., Colasurdo, G., Paul, S., and Said, S. I. (2002) *Pulm. Pharmacol. Ther.* **15**, 121-127

FOOTNOTES

[1] Abbreviations: AAU, arbitrary area unit; Ab, antibody; AMC, 7-amino-4-methylcoumarin; BCR, B cell receptor; CDRs, complementarity determining regions; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CRA, covalently reactive analog; DFP, diisopropyl fluorophosphate; EAR-MCA, Boc-Glu(OBzl)-Ala-Arg-MCA; IEGR-MCA, Boc-Ile-Glu-Gly-Arg-MCA, Fab, fragment antigen binding; FITC, fluorescein isothiocyanate; FRs, Framework regions; Ig, immunoglobulin; PE, phycoerythrin; SDS, sodium dodecylsulfate; VL and VH, light and heavy chain variable domains;VIP, vasoactive intestinal peptide.

[2] However, all 10 IgM valencies are usually not filled (*e.g.*, ref 44).

[3] If k_2, the rate constant for dissociation of the antibody-antigen noncovalent complex, is $>>k_{cat}$, the rate constant for chemical transformation of the noncovalent complex.

[4] Certain IgM Abs cleave gp120 at rates exceeding other polypeptides (S. Karle, S. Planque and S. Paul; unpublished observations).

FIGURE CAPTIONS

Fig 1. Compounds I-IV, diisopropyl fluorophosphates (DFP) and Glu-Ala-Arg-AMC. Hapten CRA I is an active site-directed inhibitor of trypsin-like enzymes. Compound II is the unesterified phosphonic acid analog of I devoid of covalent reactivity. III and IV are I-derivatives devoid of the side chain amidino function and contain a weaker leaving group, respectively. These structures are analogs of the irreversible serine protease inhibitor DFP. Boc–Glu(OBzl)-Ala-Arg-AMC is an example of a commercially available synthetic substrate in which cleavage of the amide bond between Arg and the methylcoumarinamide group releases fluorescent 7-amino-4-methylcoumarin.

Fig 2. Hapten CRA I reactivity with spleen cells. A, Flow cytometry of murine splenocytes (naïve BALB/c mouse) stained with biotinylated hapten CRA I (grey line) and compound II (black line; both compounds 100 μM, 4 hours; streptavidin-FITC (50 μg/ml). Twenty five thousand cells counted. **B,** Anti-CD19 Ab staining (grey line; phycoerythrin conjugate) of hapten CRA I labeled cells; streptavidin-FITC 1 μg/ml). (black line) shows staining with the phycoerythrin conjugate of the isotype matched control antibody. **C-F,** Deconvoluted (5 iterations) fluorescence acquisitions showing two B cells labeled with CRA I (streptavidin-FITC, 1μg/ml, panel C) and phycoerythrin conjugated anti-CD19 Ab (panel D). E shows a merged rendition of the FITC and phycoerythrin probes. F is a 3D wire frame model of the FITC emission patterns compiled from 30 individual sections and then subjected to split screen extraction. Blue counterstain, 4',6-diamidino-2-phenylindole.

Fig 3. Immunochemical identification of hapten CRA I labeled Ig subunits in B cell extracts. A, SDS-gel electrophoresis lanes showing extract of B cells labelled with hapten CRA

I (100 μM, 4 hours) following staining with silver (lane 1) and peroxidase conjugated streptavidin (lane 2). Migration of marker proteins shown on left. **B,** SDS-gel immunoblots of hapten CRA **I** labeled B cell extract stained with Abs to μ (lane 3), λ (lane 4), κ (lane 5) and γ (lane 6) chains. **C,** Streptavidin-peroxidase stained SDS-gels showing hapten CRA **I** labeled proteins recovered by affinity chromatograpy of splenocyte extract on immobilized anti-μ (lane 7), anti-κ/λ (lane 8) and anti-γ Abs (lane 9).

Fig 4. Irreversible hapten CRA I binding to IgM and IgG Abs. A, Progress curves for polyclonal murine Ab-CRA adduct formation. AAU, Arbitrary area units. Reaction conditions: IgM 0.2 μM or IgG 1 μM (equivalent combining concentration); hapten CRA **I** 0.1 mM. Values are sums of intensities of the H chain-CRA and L chain-CRA bands for IgM (●) and IgG (○; means of closely agreeing duplicates). **B,** Examples of reducing SDS-gel lanes showing CRA-Ab subunit adducts at 2 hours. Lanes 4 and 5: Streptavidin-peroxidase-stained blots showing adducts of IgM subunits and IgG subunits, respectively. IgM subunits stained with coomassie blue, anti-μ chain Ab and anti-κ/λ chain are shown in lanes 1, 2 and 3, respectively. **C,** Comparative initial velocities of hapten CRA **I** adduct formation at the subunits of IgM and IgG. Each point represents a different Ab. For comparison, data points corresponding to the μ and κ/λ chains of individual IgM Abs are connected. Abs studied: polyclonal human IgM, polyclonal mouse IgM, 5 monoclonal murine IgM Abs (clones 8702, 8704, 9008, 9010, 9020), monoclonal human IgM Yvo, polyclonal human IgG, polyclonal mouse IgG and 4 monoclonal IgG Abs (clones c23.4, c39.1, HP6045, HP6054). *$P < 0.05$ versus μ chain group in each case (Student's t-test, 2 tailed).

Fig 5: Proteolytic activities of IgM and IgG Abs. A, Cleavage of Glu-Ala-Arg-AMC (400μM)

by polyclonal murine IgM ( ), human IgM (■), polyclonal murine IgG (○) and polyclonal human IgG (□). IgM, 5 nM; IgG, 160 nM **B,** Inhibition of polyclonal murine IgM (5 nM) catalyzed Glu-Ala-Arg-AMC (400μM) cleavage by hapten CRA I (■, 30 μM; □, 100 μM). ●, progress curve without inhibitor. Values are means of triplicates ± s.d.

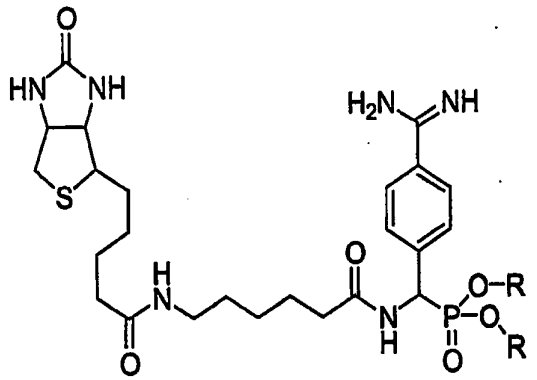
Fig 6: IgM purity. A, Purification of polyclonal murine IgM to constant specific activity. ○, IgM purified by anti-μ affinity chromatography; ●, affinity purified IgM subjected to further fractionation by FPLC gel filtration. IgM, 5 nM; Glu-Ala-Arg-AMC, 200 μM. **B,** Denaturing gel filtration profiles (Superose 12 column) of polyclonal murine IgM conducted in 6 M guanidine hydrochloride. The IgM fractions under the bar from the first cycle of denaturing chromatography (----) were pooled and subjected to 2 additional cycles of denaturing gel filtration. IgM recovered from the third chromatography cycle (—) was analyzed for catalytic activity in Panel C. **C,** Progress curve for cleavage of Glu-Ala-Arg-AMC (200μM) by IgM (2.5 nM) purified by 3 cycles of denaturing gel filtration in Panel B.

Fig 7: Divergent substrate selectivities of monoclonal IgM Abs. Data are expressed $V_{i,\ \text{Glu-Ala-Arg-AMC}}/V_{i,\ \text{Ile-Glu-Gly-Arg-AMC}}$, where V_i represents initial velocity computed from progress curves. Substrates, 200 μM. Designations 8702, 8704, 9008, 9010, 9020 and Yvo indicate the individual IgM Abs (5nM). *, IgM Yvo did not cleave Ile-Glu-Gly-Arg-AMC detectably (<0.0125 μM AMC).

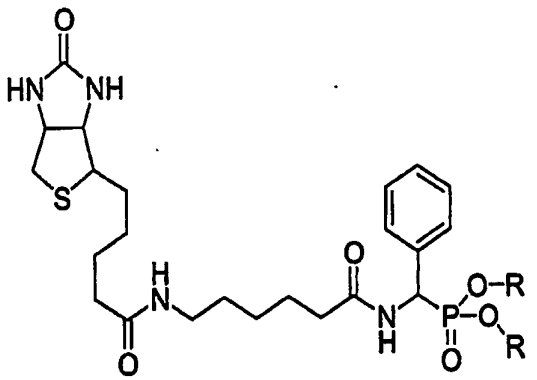
Fig 8: Cleavage of Glu-Ala-Arg-AMC by IgM Fab fragments. A, Gel filtration profile (Superose 12) of IgM Yvo without (---) and with (—) digestion with immobilized pepsin. **Inset,** Silver stained nonreducing (lane 1) and reducing (lane 2) SDS gels of the 55 kD Fab fragments. The higher and lower Mr fragments in the reducing lane correspond to the Fab heavy chain

fragment and light chain component. **B,** Progress curves of Glu-Ala-Arg-AMC (400 μM) cleavage at 1.2 μM( ), 0.4 μM (△) and 0.12 μM(■) Fab.

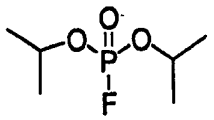
Fig 1



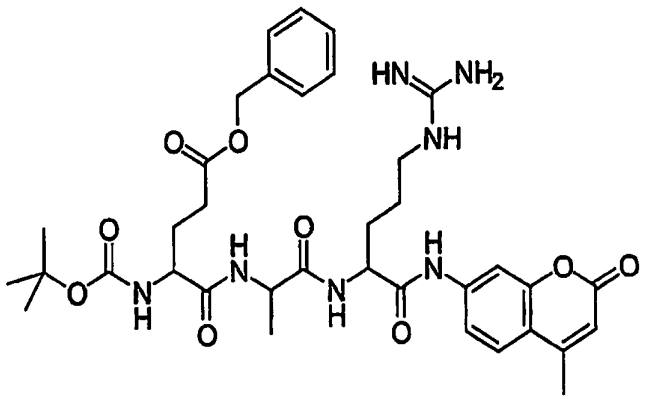
I: R = -C_6H_5
II: R = -H



III: R = -C_6H_5
IV: R = -CH_3



DFP



Boc-Glu(O-Bzl)-Ala-Arg-MCA

Fig 2

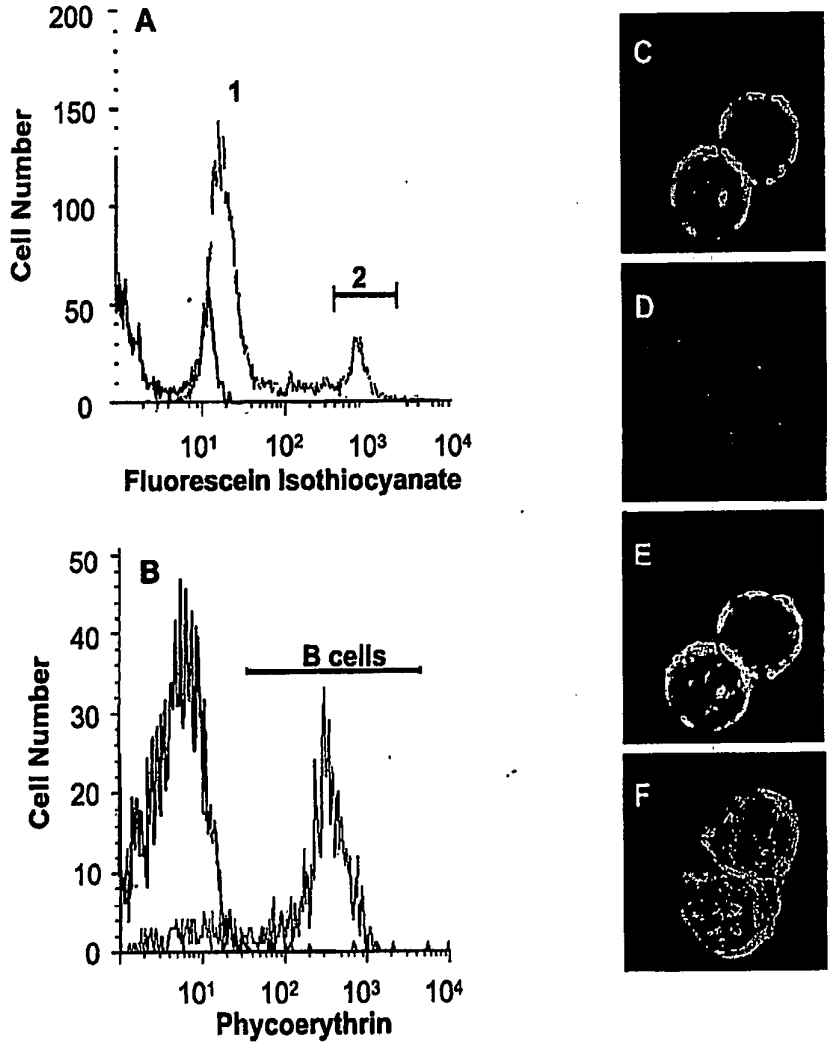

A
200
150
100
50
0
Cell Number
1
2
10^1 10^2 10^3 10^4
Fluorescein Isothiocyanate
B
50
40
30
20
10
0
Cell Number
B cells
10^1 10^2 10^3 10^4
Phycoerythrin
C
D
E
F

Fig 3

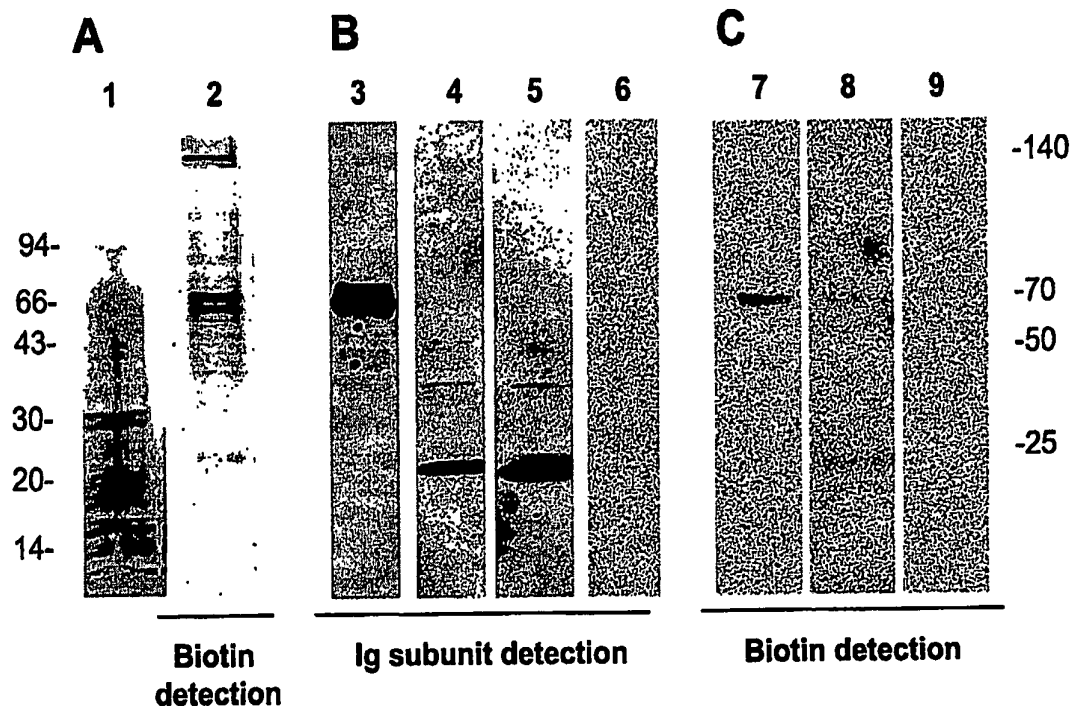

A
B
C
1
2
3
4
5
6
7
8
9
94-
66-
43-
30-
20-
14-
-140
-70
-50
-25
Biotin detection
Ig subunit detection
Biotin detection

Fig 4

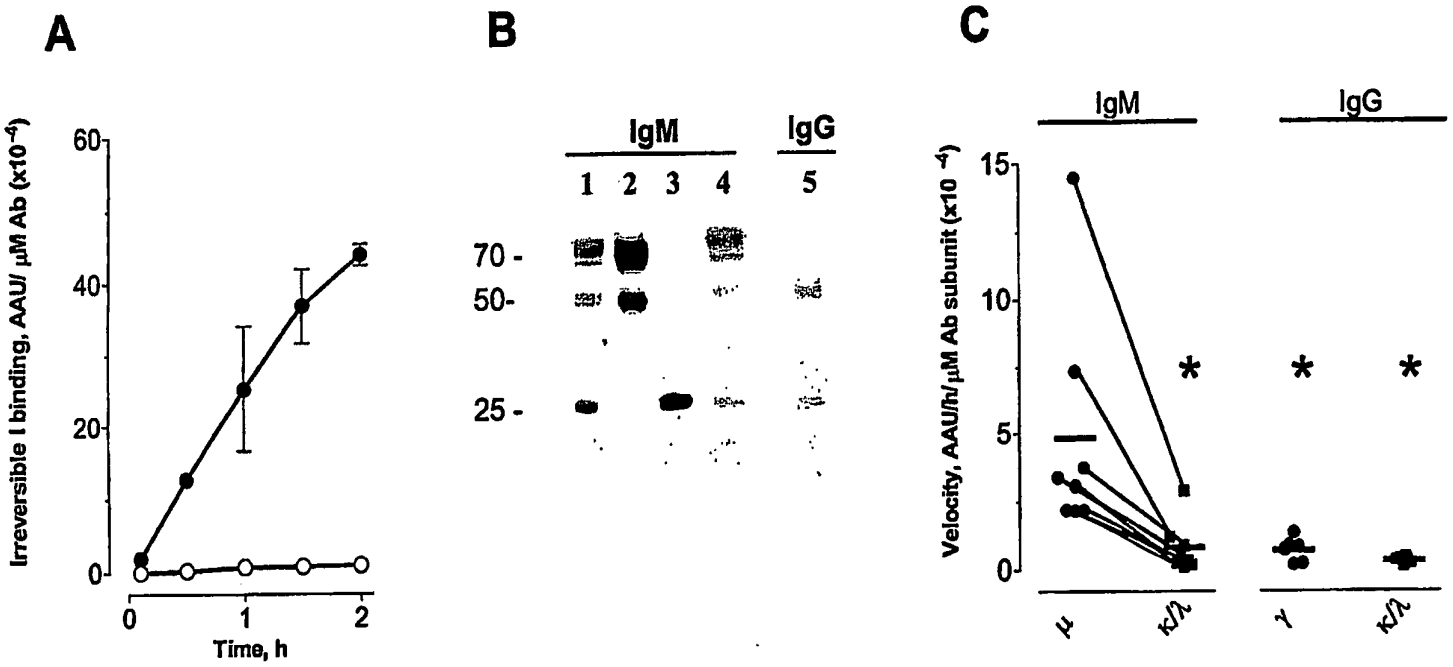

A
Irreversible I binding, AAU/ µM Ab (x10⁻⁴)
60
40
20
0
0
1
2
Time, h
B
IgM
IgG
1 2 3 4
5
70 -
50-
25 -
C
IgM
IgG
Velocity, AAU/h/µM Ab subunit (x10⁻⁴)
15
10
5
0
*
*
*
μ
κ/λ
γ
κ/λ

Fig 5

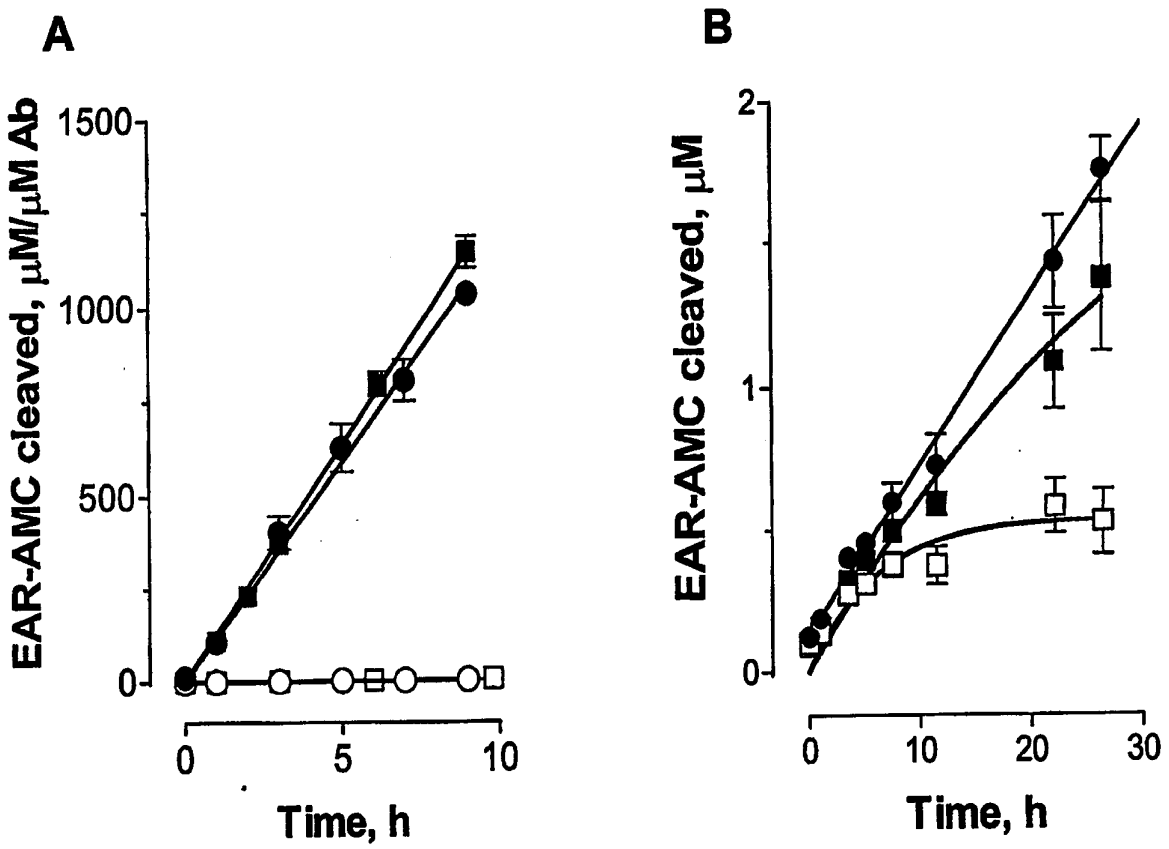

A
EAR-AMC cleaved, μM/μM Ab
1500
1000
500
0
0
5
10
Time, h
B
EAR-AMC cleaved, μM
2
1
0
0
10
20
30
Time, h

Fig 6

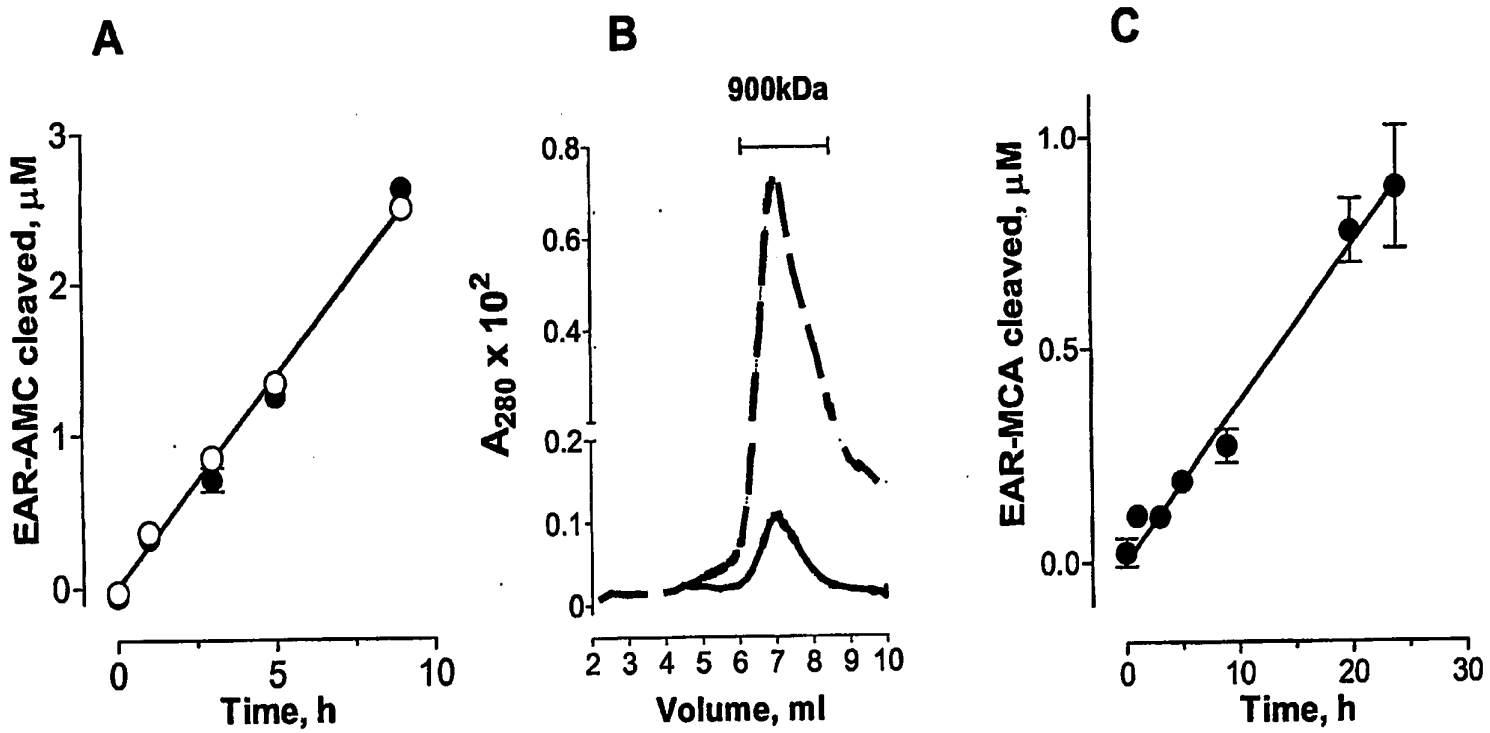

A
EAR-AMC cleaved, µM
3
2
1
0
0
5
10
Time, h
B
900kDa
A_{280} x 10^2
0.8
0.6
0.4
0.2
0.1
0
2 3 4 5 6 7 8 9 10
Volume, ml
C
EAR-MCA cleaved, µM
1.0
0.5
0.0
0
10
20
30
Time, h

Fig 7

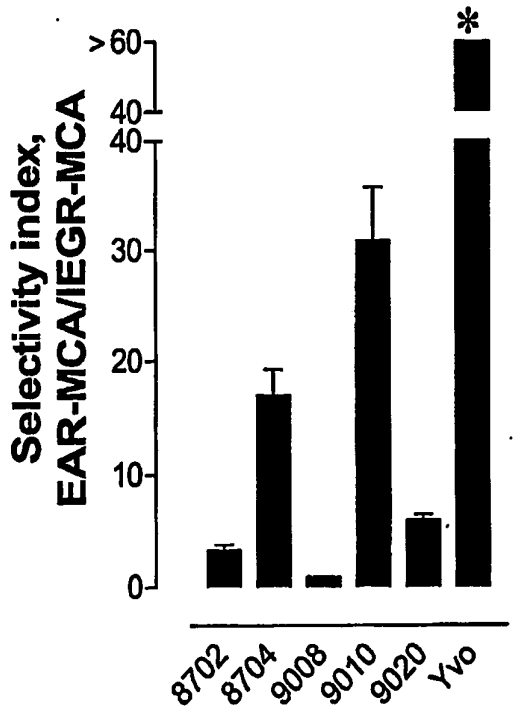
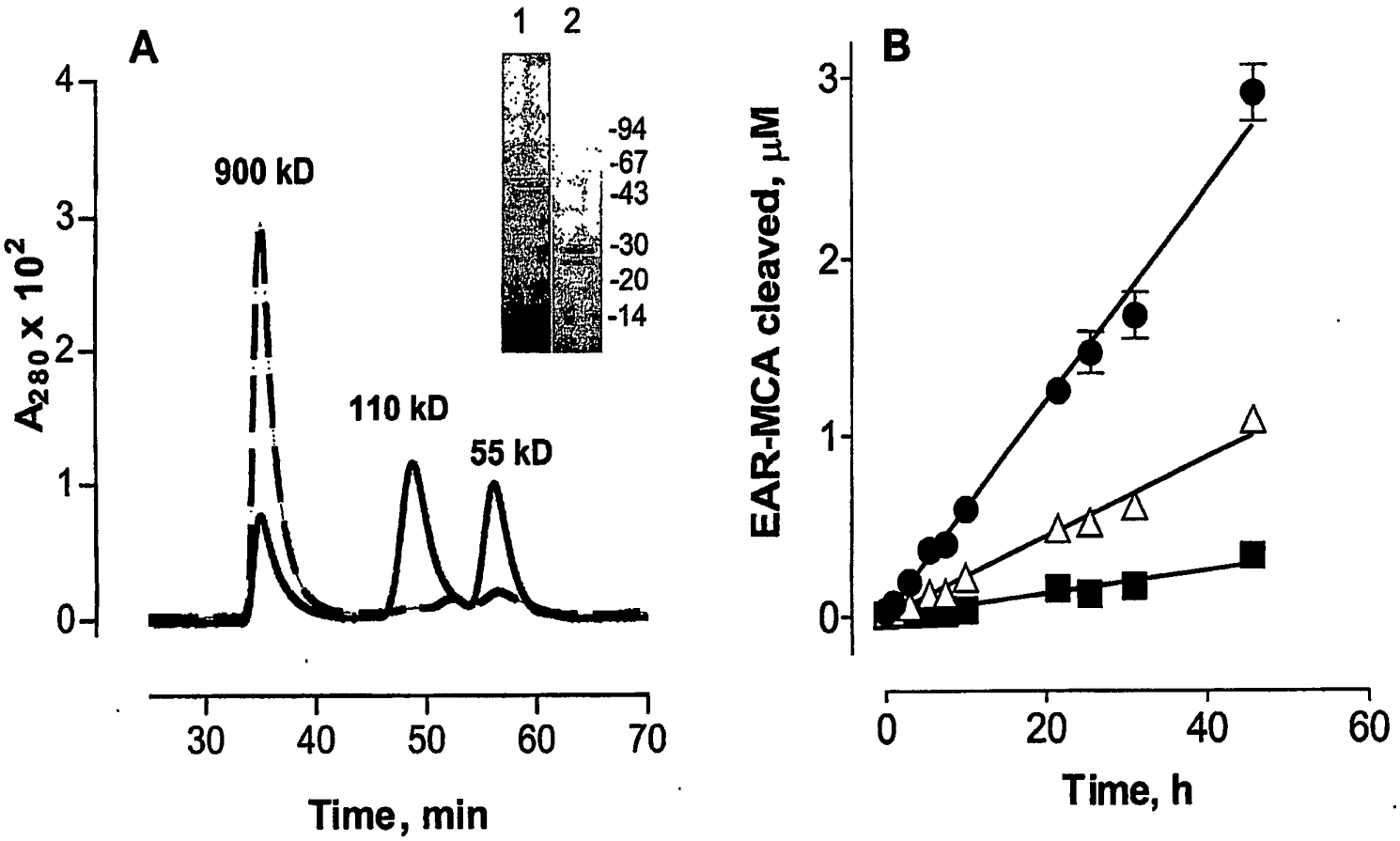

Selectivity index,
EAR-MCA/IEGR-MCA
>60
40
40
30
20
10
0
*
8702
8704
9008
9010
9020
Yvo

Fig 8


A
1 2
-94
-67
-43
-30
-20
-14
900 kD
110 kD
55 kD
A_{280} x 10^2
0
1
2
3
4
30
40
50
60
70
Time, min
B
EAR-MCA cleaved, μM
0
1
2
3
0
20
40
60
Time, h

Additional Information for Reviewers (Two figures with legends + one preprint)

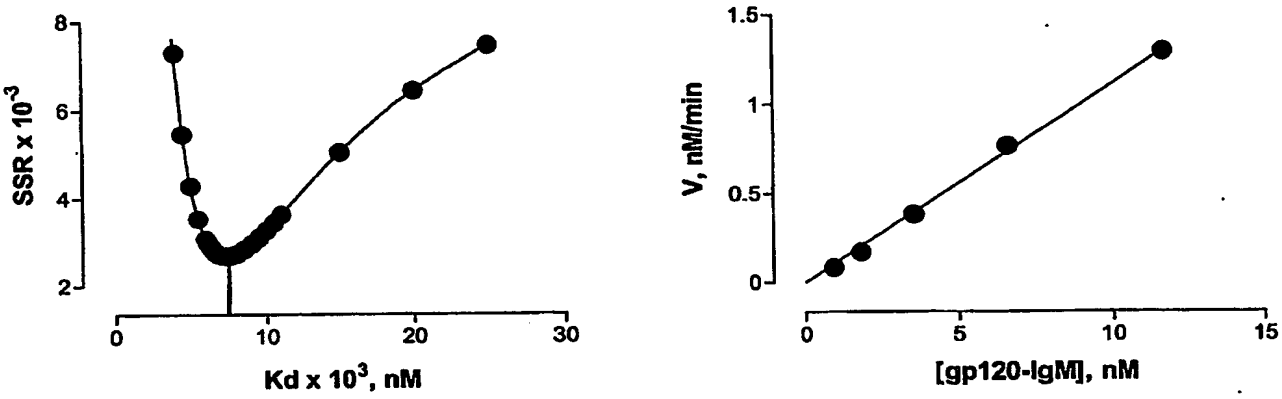


Fig 1 legend. Example of determination of kinetic parameters reported in Table 2 (IgM 8704 catalyzed gp120 cleavage).
A, Plot of Kd vs sum of square residuals (SSR). Kd, 7.4 x 10^{-6} M.
B, Plot of initial velocity vs [gp120-IgM] complex at the Kd value corresponding to the lowest SSR value obtained in panel A. r^2, 0.99. Reaction conditions as in Table 2.

Additional Information for Reviewers, cont

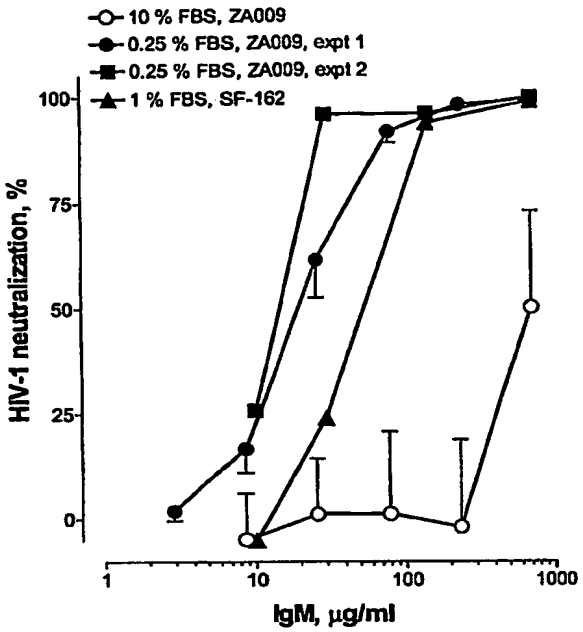


Fig 2 legend. Concentration-dependent HIV-1 neutralization by human IgM. HIV-1 neutralization by polyclonal human IgM. HIV-1 strains ZA009 and SF162 (coreceptor CCR5, clade C and B, respectively; NIH AIDS Research and Reference Reagent Program) in RPMI1640 were incubated with an equal volume of purified IgM from pooled serum of uninfected human subjects under low FBS (0.25-1 %) and customary (10 %) FBS conditions for 24 h (37°C). Phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC; 0.25 million) from healthy humans were added and the FBS concentration was adjusted to 10% in all culture wells. After further incubation for 3 days, the cells were lysed with Triton X-100, and p24 in supernatants measured by an enzymeimmunoassay kit (Beckman Coulter p24 Assay Kit; linear range 50-3200 pg/ml; see attached Karle et al preprint, *AIDS*, In press). Preliminary titrations showed that the infectivity of strains ZA009 and SF162 under low FBS conditions (0.25 % and 1%, respectively) was 4-6 fold lower compared to the customary FBS conditions (10 %). Consequently, more concentrated virus was employed in the low FBS assays to yield optimal TCID50. Values are means ± s.e.m. of 4 independent culture replicates for strain ZA009, 0.25 % FBS, experiment 1 and strain ZA009, 10 % FBS. Remaining data points obtained by measuring p24 in pooled extracts of 4 culture wells.

Comment. Treatment of strain ZA009 with human polyclonal IgM in 10% FBS-containing diluent yielded marginal effects on infection of PBMCs. FBS is customarily included in the diluent to stabilize the virus and maintain its infectivity. However, the neutralization assays are subject to potential interference by proteins present in FBS (bovine IgM, IgG and naturally occurring serine protease inhibitors). Therefore, we also conducted the virus-IgM incubations under reduced FBS conditions. In these assays, the FBS concentration was restored to 10% upon addition of the PBMC hosts (infection step). Reproducible neutralization of strains ZA009 and SF162 by the IgM was evident under these conditions.

Observed neutralization of HIV by the IgM suggests a protective role for the Abs. Possible explanations for the near-absent IgM neutralizing activity in serum-containing diluent are: (a) weakened noncovalent gp120 recognition (for example, due to FBS-induced changes in the gp120 conformation); (b) inhibition of IgM proteolytic activity by a serine protease inhibitor(s) present in FBS; or (c) masking of IgM neutralizing activity due to background neutralization by endogenous antibodies contained in FBS.

Table 1: Cleavage preference of IgM Abs. Designations 8702, 8704, 9008, 9010 and 9020 refer to murine monoclonal IgM Abs. Yvo is a human monoclonal IgM. Reaction conditions: IgM, 5 nM; peptide-AMC substrates, 200μM, except for polyclonal murine IgM (400 μM); 37°C. Blocking groups at the N termini of the substrates were: succinyl, AE-AMC, AAA-AMC, AAPF-AMC, IIW-AMC; *t*-butyloxycarbonyl, EKK-AMC, VLK-AMC, IEGR-AMC, EAR-AMC. Values (means of 3 replicates ± S.D.) are the slopes of progress curves monitored for 24 h.

Substrate	μM AMC/ h/ μM Ab							
	Polyclonal, human	Polyclonal, murine	Yvo	8702	8704	9008	9010	9020
AE-AMC	ND	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
AAA-AMC	ND	N.D.	N.D.	N.D	N.D.	.N.D.	N.D.	N.D.
IIW-AMC	ND	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
AAPF-AMC	ND	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
EKK-AMC	ND	N.D.	N.D.	0.7±0.2	6.6 ± 0.3	10.7 ± 0.8	13.1 ± 1.4	4.2 ± 0.5
VLK-AMC	1.6±0.1	N.D.	2.6±0.2	N.D.	8.0 ± 0.1	7.4 ± 0.8	6.9 ± 0.4	3.0 ± 1.7
EAR-AMC	35.4±0.7	86.4 ± 12.2	7.4±0.3	2.6 ± 0.3	18.6 ± 1.8	15.7 ± 0.6	61.6 ± 6.9	24.7 ± 1.4
IEGR-AMC	0.8±0.1	N.D.	N.D.	0.8 ± 0.2	1.1 ± 0.4	17.5± 3.2	2.0 ± 0.7	4.1 ± 0.6
PFR-AMC	5.6±0.2	20.5 ± 4.4	N.D.	N.D.	2.2 ± 0.1	6.0± 0.3	37.4 ± 0.7	13.0 ± 1.2
GP-AMC	ND	NT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

ND, not detectable (<0.125 μM AMC/h / μM Ab); NT, not tested.

Version Nov 24, 2003

Selective IgM-Catalyzed Hydrolysis of HIV gp120: An innate defense against gp120?

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SUMMARY

IgM from the serum of uninfected humans and mice selectively catalyzed the cleavage of the HIV coat protein gp120. Cleavage rates for polyclonal IgM from 5 human sera and 6 monoclonal IgMs varied over a broad range, and the Fab fragment of an IgM Ab cleaved gp120, indicating the variable domain location of the catalytic site. A monoclonal IgM cleaved gp120 at several sites, one of which was the Lys432-Ala433 peptide bond. A covalently reactive analog (CRA) of gp120 residues 421-431 extended with a C terminal amidino phosphonate diester mimic of the Lys432-Ala433 bond inhibited the catalytic cleavage of gp120. Specific covalent peptidyl CRA-IgM adducts were formed. These observations suggest a serine protease mechanism guided by noncovalent recognition of gp120 residues 422-433, and support a role for catalytic IgM antibodies as innate anti-gp120 defense enzymes.

INTRODUCTION

Hapten-like covalently reactive antigen analogs (CRAs) containing an electrophilic phosphonate diester group are reported to bind irreversibly to the variable (V) domains of IgG antibodies (Abs) suggesting the presence of enzyme-like nucleophilic sites (Planque et al., 2003). Noncovalent Ab-antigen interactions guide the nucleophilic reactivity to individual polypeptide antigens, as judged from the specific reactivity of polypeptide CRAs with Abs directed to the polypeptide component (Planque et al., 2003). Despite these properties, IgG Abs only express low-level proteolytic activities. This may be due to physiological barriers resulting from the opposing events occurring in Ab catalysis and the later stages of B cell differentiation. Occupancy of the B cell receptor complex (BCR; membrane bound Ig subunits together with noncovalently associated signal transducing proteins) by the antigen drives cell division. Efficient catalysis, on the other hand, entails rapid release of antigen fragments, which could result in cessation of antigen-driven clonal selection.

The human immunodeficiency virus (HIV) coat protein gp120 initiates viral infection by binding host cell CD4 receptors. In addition, monomer gp120 is shed from the viral and infected cell surfaces in soluble form. Free gp120 may be important in the pathogenesis of AIDS. Binding of the protein to infected CD+ cells has been implicated in depletion of T cells (Siliciano, 1996), and free gp120 also induces neuronal damage (Kaul and Lipton, 1999). Presently, no effective immunotherapeutic or vaccination strategies against HIV infection or soluble gp120 are available. The humoral IgG responses to HIV infection are generally dominated by Abs to the mutable regions of gp120. These Abs are ineffective against viral escape mutants appearing over the course of infection. Abs to the CD4 receptor binding site of gp120, developed by experimental

immunization (He et al., 2002) and phage library protocols (Burton et al., 1994), have been proposed as immunotherapy candidates. Recently, immunization with the CRA derivative of gp120 was shown to induce the synthesis of specific IgG Abs that cleave gp120 (Paul et al., 2003). Proteolytic Abs with the appropriate gp120 recognition specificity are advantageous for the purpose of permanent inactivation of the protein. Moreover, repeated reaction cycles should result in the fragmentation of multiple gp120 molecules by a single catalyst molecule. Conventional Abs bind gp120 stoichiometrically, and dissociation of the Ab-antigen complex releases the biologically active protein.

IgG Abs from healthy humans and immunologically naïve mice are reported to cleave short peptide substrates promiscuously (Kalaga et al., 1995). Reactions like esterolysis and hydrogen peroxide synthesis are also catalyzed by certain Abs regardless of the adaptive maturational status of the V domains (Wentworth et al., 2000; Patten et al., 1996). As the first class of Abs synthesized in B cell development, IgM Abs usually contain V domains with lower levels of adaptive sequence diversification than IgG V domains produced by differentiated B cells. In the course of studies on Ab-catalyzed gp120 cleavage (Paul et al., 2003), we discovered the phenomenon of selective and efficient gp120 cleavage by IgM from the preimmune repertoire. The IgM Abs recognize a peptide determinant reported to contribute contact sites for CD4, i.e., gp120 residues 421-433 (Goodglick et al., 1995; Karray and Zouali, 1997). This determinant is also implicated in gp120 recognition as a superantigen by the conserved V domain regions of certain Abs (Neshat et al., 2000; Karray et al., 1998).

RESULTS

Catalytic activity of polyclonal IgM Abs. Biotinylated gp120 (Bt-gp120) was cleaved by IgM from all 5 human sera (Fig 1A), assessed by depletion of the parent gp120 band and appearance of fragments with lower mass in electrophoresis gels (Fig 1B; the recombinant protein migrates with Mr ~94 kD, presumably because of incomplete glycosylation in the baculovirus expression system). None of the IgG samples from the 5 human sera cleaved gp120 detectably. The data in Fig 1A are expressed per equivalent combining sites of IgM and IgG (10 and 2, respectively; note, however, that all 10 IgM valencies are usually not filled (Chavin and Franklin, 1969). Superior IgM catalysis, therefore, can not be ascribed to the greater number of IgM combining sites. Essentially identical results were obtained using IgM and IgG Abs prepared from the pooled sera of immunologically unstimulated BALB/c mice (87.9% cleavage/20hours/150 nM IgM combining sites; undetectable gp120 cleavage at equivalent IgG combining site concentration; reaction conditions as in Fig 1A).

Affinity-purified IgM was subjected to two cycles of gel filtration in the denaturing solvent, 6 M guanidine hydrochloride (Fig 2). Abs recovered from this procedure were renatured by dialysis and the catalytic activity was measured. IgM subjected to denaturing gel filtration displayed near-equivalent Bt-gp120 cleaving activity as the starting IgM fraction (82.2 ± 9.2 and 78.8 ± 1.0 % cleavage, respectively), indicating the absence of noncovalently-associated contaminating proteases.

Catalytic activity of monoclonal IgM Abs. Each of six monoclonal IgM Abs studied (5 murine; 1 human) displayed gp120 cleaving activity (Fig 3A). IgM Yvo purified by repetitive warming

and cooling was analyzed for catalytic activity prior to and after further affinity chromatography on an anti-IgM column. The levels of gp120 cleavage before and after the final purification step were essentially identical (46.4 ±0.6 % (s.d.) and 44.3 ± 4.9% Bt-gp120 cleaved/50 nM IgM/6 hours, respectively; reactions as in Fig 1B). This fulfils the criterion of purification to constant specific activity required for assignment of enzymatic activity to IgM.

Identically-purified murine IgM Abs expressed differing levels of catalytic activity varying over a 27.5-fold range; Fig 3A). As the constant domain architecture of the Abs is identical, the results suggest that the catalytic activity is a V domain property. This is supported by observation of gp120 cleaving activity in the Fab fragment of IgM Yvo prepared by digestion with immobilized pepsin and gel filtration (Fig 3B). Under the neutral pH conditions employed, pepsin is inactivated irreversibly (Cornish-Bowden et al., 1969). Treatment of Bt-gp120 with purified pepsin (1.2 μM) did not cleave the protein detectably (not shown; reaction conditions as in Fig 3C), confirming that the observed gp120 cleaving activity of Fab is not due to pepsin contamination.

Treatment of Bt-gp120 with IgM Yvo generated an 80 kD biotin-containing fragment, the intensity of which changed only marginally over the course of the reaction, accompanied by progressive disappearance of the parent band (Fig 4A). This suggested that the 80 kD band may be susceptible to additional cleavage reactions. Several faint biotin-containing bands appeared at lower Mr values in highly overexposed gels (43, 31, 25 and 17 kD; not shown). Because of the low biotin content of gp120 (1.4 moles/moles), the presence of biotin does not accurately predict the relative abundance of the fragments. Incubation of gp120 devoid of biotin with IgM Yvo generated several coomassie blue-stainable bands at 15.4 -18.8 kD (Fig 4B; bands at 25 kD and

70 kD correspond to IgM Yvo light and heavy chains, respectively). The fragments were subjected to N terminal amino acid sequencing (Table 1). The 80 kD gel region yielded a sequence corresponding to the N terminus of gp120. As no internal gp120 peptide sequence was detected, the cleavage site was not identifiable, but it was concluded that the fragment is produced by cleavage at a site located in the C terminal half of gp120. Peptide sequences corresponding to gp120 residues 433-442 were identified in fragments corresponding to 15.4-16.4 kD and 17.6 kD gel regions, permitting unambiguous identification of the 432-433 peptide bond as one of the cleavage site. Two peptide sequences corresponding to gp120 residues 1-10 were present in the 15.4-16.4 kD and 18.8 kD gel regions. These gp120 fragments indicate the existence of two additional cleavage sites located in the N terminal half of the protein.

Kinetics and selectivity. Treatment of biotinylated bovine serum albumin (Bt-BSA), biotinylated soluble CD4 (Bt-sCD4) or biotinylated soluble epidermal growth factor receptor (Bt-sEGFR) with polyclonal human IgM did not result in noticeable depletion of the electrophoresis bands corresponding to full-length form of these proteins (Fig 5). Under these conditions, readily detectable Bt-gp120 cleavage was observed. Similarly, there was no detectable depletion of full-length Bt-sCD4, Bt-sEGFR and Bt-BSA bands treated with IgM Yvo and polyclonal murine IgM (reaction conditions as in Fig 5; cleavage of Bt-gp120 by these Abs, 69.1 % and 97.4 % respectively).

The kinetic parameters for three IgM preparations were determined by fitting the observed initial rates at increasing concentrations of gp120 to the general quadratic equation predicting the concentration of the catalyst-substrate complex (Table 2). The method is is applicable to the study of the reaction kinetics when the gp120 concentrations are lower than the K_d. The apparent K_d is

the value yielding the best fit between observed velocity and [IgM-gp120], and apparent k_{cat} is the slope of the velocity versus [IgM-gp120] plot. Correlation coefficients for the 3 plots corresponding to the data reported in Table 2 were >0.9. Observed k_{cat} values for polyclonal human IgM, monoclonal human IgM Yvo and monoclonal murine IgM 8704 varied over a 52.5-fold range, and the K_d values, over a 23.8-fold range.

Nucleophilic reactivity. The phosphonate diester-containing analog of gp120 residues 421-432 (gp120pep-CRA; Fig 6A) has been developed as a covalent probe for nucleophilic anti-gp120 Abs. Abs raised by immunization with the peptide component bind irreversibly and rapidly with gp120pep-CRA due to the facilitatory effects of noncovalent antigen-Ab binding interactions (Planque et al., 2003). In the present study, progressive inhibition of the cleavage of Bt-gp120 by IgM Yvo was observed at increasing gp120pep-CRA concentrations (Fig 6B). Covalent gp120pep-CRA binding was measured by estimating the biotin content in protein adduct bands on electophoresis gels. All 5 IgM Ab preparations studied (3 monoclonal IgMs, murine polyclonal IgM and human polyclonal IgM) formed covalent adducts with the gp120pep-CRA at rates exceeding adduct formation with hapten CRA **I** (Fig 7A; mean rate, 41-fold greater for the former compound; $P < 0.002$, Student's t test, 2 tailed). The μ chain subunit accounted for the majority of covalent gp120pep-CRA binding except in the case of monoclonal IgM Yvo (Fig 7B; rates in arbitrary area units (AAU)/h/μM Ab subunit: H chain, 41.5–257.5; L chain, 22.3–247.7). The CRA adducts accumulated linearly as a function of time (e.g., IgM Yvo L chain adducts shown in Fig 7C). Inclusion of excess synthetic gp120(421-436) in the reaction mixture (500 μM) inhibited the formation of gp120pep-CRA adducts by each of the 5 IgM preparations by ≥ 68%, regardless of the subunit at which the reaction occurred (e.g., Fig 7D).

DISCUSSION

These studies indicate the selective ability of IgM Abs to catalyze the cleavage of the HIV coat protein gp120. The Abs were from uninfected humans and immunologically naïve mice. IgG Abs, products of B cells at more advanced stages of differentiation, did not cleave gp120 appreciably. The observed selectivity of IgM catalyzed gp120 hydrolysis, therefore, must reflect the intrinsic properties of the preimmune Ab repertoire. Assignment of the catalytic activity to IgM V domains is supported by these observations: (a) IgM Abs differing only by virtue of their V domains displayed divergent levels of catalytic activity; (b) The Fab fragment expressed the activity; and (c) Formation of covalent IgM adducts with the gp120 pep-CRA probe proceeded more rapidly than the hapten CRA; different Abs formed the adducts at varying levels; the reaction for different Abs displayed distinct subunit preferences; and, the reaction was inhibited by a synthetic peptide spanning the peptide determinant located in gp120pep-CRA. The CRA phosphonate diester group inhibits serine proteases by covalent binding at the activated Ser nucleophile (Oleksyszyn and Powers, 1994; Sampson and Barton, 1991). A germline configuration light chain has been described to express serine protease-like proteolytic activity (Gololobov et al., 1999) and IgG Abs in the preimmune murine and human repertoires are also reported to express proteolytic activities (Kalaga et al., 1995; Matsuura et al., 1998). The reactivities of proteolytic IgMs described here are consistent with the germline origin of the catalytic activity.

Avidity effects due to the decavalent character of IgM can strengthen the binding to antigens with repeat epitopes even if the intrinsic affinity of the individual combining sites is small. Superior catalysis by IgM compared to IgG can not be attributed to this factor, however, as gp120 does not

contain repeat epitopes. Furthermore, catalysis assays conducted in solution using monoclonal Abs do not favor multivalent IgM binding to the same gp120 molecule. The following explanations can be presented for the superior catalytic activity of IgM Abs. First, loss of catalytic activity may be attendant to V domain somatic diversification after isotype switching from IgM to IgG. Second, distinctive IgM constant domain characteristics may be important in maintaining the integrity of the catalytic site, in which case isotype switching itself may result in reduced catalytic activity. These explanations are not mutually exclusive. Both explanations are consistent with the argument that catalysis is a disfavored phenomenon in the advanced stages of B cell development, as efficient BCR catalysis is predicted to result in reduced BCR occupancy. We did not attempt to address these points experimentally in the present study. However, the monovalent Fab studies suggested that disruption of the constant domain architecture of IgM is deleterious for catalysis. The Fab preparations displayed ~100-fold lower activity than computed for the individual combining sites of pentameric IgM. Pepsin employed to prepare Fab cleaves μ chains on the C terminal side of the CH2 domain (MacKenzie et al., 1978), which is distinguished by its conformational flexibility (Roux et al., 1998). Alterations of antigen binding activity when the same V domains are expressed as full-length IgG Abs belonging to different isotypes are described (e.g., Morelock et al., 1994), but we are not aware of IgM-IgG V domain swapping experiments in the literature. Positive cooperativity effects such as those described for antigen binding by the two IgG combining sites (van Erp et al., 1991), could theoretically furnish favorable contributions in catalysis. The sequence of events as individual IgM combining sites bind antigen has not been elucidated, but the hypothesis of positive cooperativity is not supported by findings that only 5 of the 10 IgM combining sites are filled at excess antigen concentration (e.g., Chavin and Franklin, 1969).

Selective gp120 recognition by IgM proteases can not be understood from the local chemical interactions confined to recognition of the dipeptide cleavage site, as the same dipeptide units are present in other poorly cleaved proteins. The selectivity probably arises from noncovalent gp120 recognition by Abs. This may be deduced from the comparatively small K_d values for catalytic IgM recognition of gp120, 1.3-30.0 μM. These values are about 2 orders of magnitude smaller than the apparent K_d for promiscuous proteolytic reactions catalyzed by IgM Abs (S. Planque and S. Paul, unpublished observations) and IgG Abs isolated from the sera of preimmune mice and healthy humans (Kalaga et al., 1995). Further support for the importance of noncovalent interactions can be drawn from observations that the covalent reaction of gp120pep-CRA with IgM Abs is guided by noncovalent recognition of its peptide component. Precedents for Ab catalytic selectivity derived from noncovalent recognition are available. Noncovalent paratope-epitope binding coordinated with nucleophilic attack on the scissile bond is the basis for selective cleavage of individual polypeptide antigens by adaptively matured proteolytic Abs obtained by experimental immunization (Paul et al., 2003; Sun et al., 1997).

Selective cleavage of gp120 by IgM from subjects not infected with HIV may be traced to the superantigenic character of gp120 (Berberian et al., 1993). IgM Abs are described to bind gp120 as a superantigen (Townsley-Fuchs et al., 1996; Juompan et al., 1998) by contacts at conserved Ab V domain regions (Neshat et al., 2000; Karray et al., 1998). The superantigenic site of gp120 consists of discontinuous peptide segments, one of which spans residues 421-433 (Goodglick et al., 1995; Karray and Zouali, 1997). An IgM Ab studied here cleaved the peptide bond linking residues 432 and 433. All of the catalytic IgM Abs displayed selective covalent binding of gp120pep-CRA, which contains residues 421-431 and an amidino phosphonate mimetic of residues 432 and 433. This supports a model entailing noncovalent gp120 binding that is

functionally coordinated with the nucleophilic reactivity of the catalytic site. The proposed mechanism is identical to that utilized by catalytic IgG Abs induced by experimental immunization (Paul et al., 2003; Sun et al., 1997), except that the noncovalent binding takes place at conserved V domain regions instead of the adaptively matured hypervariable loops. However, certain aspects of the model remain to be explored. For instance, the covalent gp120pep-CRA data suggest the extent to which the nucleophilic reactivity is coordinated with noncovalent peptide epitope recognition, but they do not establish the subunit location of the catalytic nucleophile. Adducts of gp120pep-CRA were formed mainly by the heavy chain of four IgM preparations and the light chain of one IgM preparation. Previous reports indicate that the light and heavy chains can each express catalytic nucleophiles (Gao et al., 1995; Matsuura and Sinohara, 1996; Hatiuchi et al., 2003). Another interesting aspect is the cleavage of multiple peptide bonds in gp120, analogous to the complex cleavage profiles reported for monoclonal Ab L chain catalyzed fragmentation of gp41 (Hifumi et al., 2002) and vasoactive intestinal peptide (VIP) (Sun et al., 1997). The fragmentation profiles may be explained by the formation of alternate Ab-gp120 ground state complexes with different peptide bonds positioned in register with the nucleophilic residue (Paul et al., 2003). When the Ab recognizes a conformational epitope, the alternate cleavage sites must be spatially adjacent but they can be distant in the linear sequence, producing complex cleavage pattern.

Noncovalent IgM-gp120 complexes reported previously contain Abs with VH domains belonging to the VH3 family (Goodglick et al., 1995; Karray and Zouali, 1997; Berberian et al., 1993). The VH domain of IgM Yvo belongs to the VH2 family (VH2-5 germline gene; deduced from Shaw et al., 2002; VH sequences of remaining IgM Abs studied here are not available). This discrepancy may be explained as follows. First, proteolysis of gp120 entails rapid product release.

Efficient catalysts will be detected poorly by binding assays because of the small concentration of stable immune complexes. Second, the turnover capability allows more sensitive detection of catalysts than noncatalytic Abs. At the IgM (15 nM) and gp120 concentrations (100 nM) in Fig 1A, a noncatalytic Ab with K_d 31 μM will bind only 0.5 nM gp120 at equilibrium [computed from the equation $[Ab\text{-}Ag]^2-[Ab\text{-}Ag]([Ab_0]+[Ag_0]+K_d)+[Ab_0][Ag_0]=0$, where $[Ab_0]$and $[Ag_0]$ are Ab and antigen concentrations at time 0]. In comparison, 70 nM gp120 will be cleaved over 20 hours under similar conditions by a catalytic IgM preparation with k_{cat} 2.1/min and K_d equivalent to the noncatalytic Ab (computed as $P_t=Ag_0[1-e^{(-k[Ab_0]t)}]$, where P_t is product concentration at time t and k is k_{cat}/K_m; Marangoni, 2003).

Catalytic Abs produced spontaneously by the immune system have been viewed until now primarily as pathogenic effector molecules, e.g., autoantibodies to VIP (Paul et al., 1989), nucleic acids (Shuster et al., 1992) and Factor VIII (Lacroix-Desmazes et al., 1999). The present study suggests that IgM catalysis may be relevant to the pathogenesis of HIV infection. Free gp120 shed from HIV is thought to exert deleterious effects on several cell types. The neurotoxic effect of free gp120 has been implicated in AIDS dementia (Kaul and Lipton, 1999) and its ability to induce apoptosis may contribute to the decline of CD4+ T cells, regardless of whether the cells are infected (Siliciano, 1996). A caveat in assessing the functional potency of IgM Abs is the possibility of inhibition by naturally occurring serine protease inhibitors in blood and other anatomic locations relevant to HIV infection. In the absence of inhibitors, circulating human IgM at 2 mg/ml in blood may be computed to hydrolyze 50% and 90% of gp120 present at concentrations $<< K_d$ in 4.6 min and 15.5 min, respectively (assuming K_d 31 μM, k_{cat} 2.1/min, Table 2). Similarly, if cleavage of trimeric gp120 on the viral surface proceeds at the rate observed for the free protein, only short time periods are needed to hydrolyze the majority of viral

gp120 (gp120 concentrations in infection remain << observed K_d; *e.g.*, 10^6 HIV copies/ml with 100 gp120 molecules/virion correspond to ~$2x10^{-13}$ M gp120; Richieri et al., 1998). Support for a protective role for IgM Abs can be drawn from their ability to recognize gp120 residues 421-433. These residues contribute contact sites in the binding of gp120 by host cell CD4 receptors (Olshevesky et al., 1990; Kwong et al., 1998). Fragments generated by cleavage at the IgM-sensitive Lys432-Ala433 bond are reportedly devoid of CD4 binding activity (Pollard et al., 1991). IgG Abs that bind the gp120 superantigenic site noncovalently are described as resistance factors in progression of HIV infection (Townsley-Fuchs et al., 1996; Juompan et al., 1998). Initial studies conducted as in Karle et al., In press suggest that polyclonal human IgM can neutralize the infection of peripheral blood mononuclear cells by primary HIV-1 isolates under low serum conditions (Hanson, Karle and Paul, to be published elsewhere). Berberian et al., 1993 have previously cited their unpublished data suggesting that HIV neutralization in the absence of serum by IgM antibodies that bind the superantigenic site gp120 (citation 11 in Berberian et al., 1993).

These studies may also be relevant to HIV vaccine design. Synthetic peptides containing gp120 residues 421-433 have been advanced as vaccine candidates (Morrow et al., 1992; Karle et al., 2003), in part because these residues are comparatively conserved in diverse HIV strains. The gp120 peptidyl CRA described here is a potential immunogen for induction of Abs with strengthened recognition of the gp120 superantigenic site. A CRA derivative of full-length gp120 induces the synthesis of catalytic Abs (Paul et al., 2003), but Abs to irrelevant epitopes probably dominate the response to this immunogen.

EXPERIMENTAL PROCEDURES

Antibodies. Human serum Abs were isolated from subjects without evidence of infection or immunological disease (2 females, 3 males; age 23-45 yrs). Murine serum Abs were from BALB/c mice (Harlan, Indianapolis, IN; pooled from 150 mice; age 8-12 weeks). Murine monoclonal IgM Abs used here are directed against certain major histocompatibility antigens (clones corresponding to catalog nos. 8702, 8704, 9008, 9010 and 9020; cell-free ascites; Cedarlane, Ontario, Canada). Monoclonal IgM Yvo was obtained by plasmapheresis of a patient with Waldenstrom's macroglobulinemia (Shaw et al., 2002). All monoclonal IgM Abs contained κ chains. Serum or ascites (1ml) was mixed for 1h with 1 ml Sepharose 4B conjugated rat anti-mouse IgM Abs (settled gel; Zymed, San Francisco, CA) or agarose conjugated goat anti-human IgM Abs (Sigma, St. Louis, MO) with IgM binding capacities 0.8 and 3 mg, respectively, in 50 mM Tris-HCl, pH 7.5, 0.1 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid) (buffer A). The unbound fraction was recovered and the gel washed with 20 buffer A volumes, taking care that protein in the effluent had returned to undetectable levels prior to elution ($A_{280} < 0.001$). Elution was with 100 mM glycine pH 2.7 (0.5 ml/fraction into 25 µl 1M Tris-HCl, pH 9.0). Further purification was on a Superose-6 FPLC gel filtration column (1x30 cm; 0.25 ml/min; Pharmacia) in two different solvents: 50 mM Tris-HCl, pH 7.7, 0.1 M glycine, 0.15 M NaCl, 0.025% Tween-20 (buffer B) or 6 M guanidine hydrochloride in buffer B adjusted to pH 6.5 with HCl (buffer C). Prior to column fractionation, the affinity purified IgM was dialyzed against buffer C. Column calibration was with thyroglobulin (660 kD), IgG (150 kD) and albumin (67 kD). The IgM eluted with apparent Mr 900 kD close to the void volume of the column. IgM was renatured following buffer C chromatography by dialysis against buffer B (Kalaga et al., 1995). IgM Yvo, a cryoglobulin, was purified from serum by repetitive warming

(37°C) and cooling (4°C; 3 cycles; Shaw et al., 2002) followed by affinity chromatography on the anti-human IgM column. IgG was purified on Protein G-Sepharose columns (Kalaga et al., 1995) using as starting material the unbound fraction from the anti-IgM columns or cell-free ascites. Fab fragments were prepared by digesting IgM (300 µl, 1mg/ml) with agarose conjugated pepsin (0.6 ml gel, 30 min, 37°C) in 100 mM sodium acetate, pH 4.5, 150 mM NaCl, 0.05% NaN_3, 0.1 mM CHAPS) as recommended by the manufacturer (Pierce). The unbound fraction was dialyzed against buffer B, purified by FPLC gel filtration on a Superose 12 column and dialyzed against 50 mM Tris-HCl, pH 7.7, 0.1 M glycine, 0.1 mM CHAPS. Total protein was determined by the bicinchoninic acid method (Pierce). SDS-polyacrylamide gel electrophoresis (4-20% gels) was conducted under reducing conditions (2-mercaptoethanol). Blots of the gels were stained with peroxidase conjugated goat anti-human µ, γ, κ and λ Abs (1:1000; Sigma) or goat anti-mouse µ, γ, κ and λ Abs followed by peroxidase conjugated rabbit anti-goat IgG (Fc specific, 1:1000; Pierce; Kalaga et al., 1995). Nominal Mr values were computed by comparison with standard proteins (14 kDa –94 kDa; Pharmacia).

Proteolysis assays. gp120, the soluble extracellular domain of the epidermal growth factor receptor (sEGFR) and bovine serum albumin were labeled with biotin (Bt) at Lys residues has been described (1-2 mol Bt/mol protein) as described in Planque et al., 2003. Soluble CD4 (sCD4; residues 1-183; NIH AIDS Reagent Program) was biotinylated and purified by similar methods (1.3 mol Bt/mol sCD4). The gp120 (strain MN) is a recombinant protein expressed in the baculovirus system (Immunodiagnostics, Woburn, MA). Synthesis of gp120pep-CRA (Bt-KQIINMWQEVGN with the amidino phosphonate diester group at the C terminus) is described in Taguchi et al., 2002. Catalysis assays (Paul et al., 2003) were performed by incubating Bt-

proteins with the Abs in 50 mM Tris-HCl, 100 mM glycine, pH 7.7, 1 mM CHAPS at 37°C. The samples were boiled in buffer containing SDS and 2-mercaptoethanol, electrophoresed on SDS-gels. Cleavage was determined by densitometry of electroblots stained with streptavidin peroxidase. Assays for inhibition of catalysis by the gp120pep-CRA were performed in 6% ethanol. Purified porcine pepsin used as control in Fab cleavage studies was from Sigma N terminal sequencing of gp120 fragments electroblotted from electrophoresis gels was performed as in Sun et al., 1997 (Applied Biosystems Model 492 Procise cLC sequencer). Kinetic parameters were determined by fitting rate data at varying Bt-gp120 concentrations to the quadratic equation (Sun et al., 1997): $[CS]^2-[CS]([C_t]+[S_t]+K_d)+[C_t]\ [S_t]=0$, where $[C_t]$ and $[S_t]$ are the total concentrations of catalyst and substrate, and [CS] is the concentration of the catalyst-substrate complex.

Irreversible CRA binding. Synthesis of the biotin-containing hapten phosphonate CRA, its irreversible reaction with proteases and Abs, and the irreversible binding of gp120pep-CRA with specific Abs to the synthetic peptide composed of gp120 residues 421-436 have been described (Planque et al., 2003; Taguchi et al., 2002; Nishiyama et al., 2002). Formation of CRA-IgM adducts was measured by reducing SDS-electrophoresis, electroblotting and densitometry using a streptavidin-peroxidase conjugate (Planque et al., 2003). Band intensities are expressed in arbitrary area units. Initial velocities were computed as slopes of progress curves (incubation for 20, 40, 60, 120 and 220 min; $r^2 > 0.9$ for all data reported here).

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REFERENCES

Berberian, L., Goodglick, L., Kipps, T.J., and Braun, J. (1993). Immunoglobulin VH3 gene products: natural ligands for HIV gp120. Science *261*, 1588-1591.

Burton, D.R., Pyati, J., Koduri, R., Sharp, S.J., Thornton, G.B., Parren, P.W., Sawyer, L.S., Hendry, R.M., Dunlop, N., and Nara, P.L. (1994). Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science *266*, 1024-1027.

Chavin, S.I., and Franklin, E.C. (1969). Studies on antigen-binding activity of macroglobulin antibody subunits and their enzymatic fragments. J. Biol. Chem. *244*, 1345-1352.

Cornish-Bowden, A., and Knowles, J. (1969). The pH dependence of pepsin-catalyzed reactions. Biochem. J. *113*, 353-362.

Gao, Q.-S., Sun, M., Rees, A., and Paul, S. (1995). Site-directed mutagenesis of proteolytic antibody light chain. J. Mol. Biol. *253*, 658-664.

Gololobov, G., Sun, M., and Paul, S. (1999). Innate antibody catalysis. Mol. Immunol. *36*, 1215-1222.

Goodglick, L., Zevit, N., Neshat, M.S., and Braun, J. (1995). Mapping the Ig superantigen-binding site of HIV-1 gp120. J. Immunol. *155*, 5151-5159.

Hatiuchi, K., Hifumi, E., Mitsuda, Y., and Uda, T. (2003). Endopeptidase character of monoclonal antibody i41-7 subunits. Immunol. Lett. *86*, 249-257.

He, Y., Honnen, W.J., Krachmarov, C.P., Burkhart, M., Kayman, S.C., Corvalan, J., and Pinter, A. (2002). Efficient isolation of novel human monoclonal antibodies with neutralizing activity against HIV-1 from transgenic mice expressing human Ig loci. J. Immunol. *169*, 595-605.

Hifumi, E., Mitsuda, Y., Ohara, K., and Uda, T. (2002). Targeted destruction of the HIV-1 coat protein gp41 by a catalytic antibody light chain. J. Immunol. Methods. *269*, 283-298.

Juompan, L., Lambin, P., and Zouali, M. (1998). Selective deficit in antibodies specific for the superantigen binding site of gp120 in HIV infection. FASEB J. *12*, 1473-1480.

Kalaga, R., Li, L., O'Dell, J., and Paul, S. (1995). Unexpected presence of polyreactive catalytic antibodies in IgG from unimmunized donors and decreased levels in rheumatoid arthritis. J. Immunol. *155*, 2695-2702.

Karle, S., Nishiyama, Y., Zhou, Y.-X., Luo, J., Planque, S., Hanson, C., and Paul, S. (2003). Carrier-dependent specificity of antibodies to a conserved peptide determinant of gp120. Vaccine *21*, 1213-1218.

Karle, S., Planque, S., Nishiyama, Y., Taguchi, H., Zhou, Y.-X., Salas, M., Lake, D., Thiagarajan, P., Arnett, F., Hanson, C.V., and Paul, S. (2003). Cross-clade HIV-1 neutralization by an antibody fragment from a lupus phage display library. AIDS. In press.

Karray, S., Juompan, L., Maroun, R.C., Isenberg, D., Silverman, G.J., and Zouali, M. (1998). Structural basis of the gp120 superantigen-binding site on human immunoglobulins. J. Immunol. *161*, 6681-6688.

Karray, S., and Zouali M. (1997). Identification of the B cell superantigen-binding site of HIV-1 gp120. Proc. Natl. Acad. Sci. USA *94*, 1356-1360.

Kaul, M., and Lipton, S.A. (1999). Chemokines and activated macrophages in HIV gp120-induced neuronal apoptosis. Proc. Natl. Acad. Sci. USA *96*, 8212-8216.

Kwong, P.D., Wyatt, R., Robinson. J., Sweet, R.W., Sodroski, J., and Hendrickson, W.A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature *393*, 648-659.

Lacroix-Desmazes, S., Moreau, A., Sooryanarayana, B.C., Stieltjes, N., Pashov, A., Sultan, Y., Hoebeke, J., Kazatchkine, M.D. and Kaveri, S.V. (1999). Catalytic activity of antibodies against factor VIII in patients with hemophilia A. Nat. Med. *5*, 1044-1047.

MacKenzie, M.R., Gutman, G.A., and Warner, N.L. (1978). The binding of murine IgM to staphylococcal A protein. Scand. J. Immunol. *7*, 367-370.

Marangoni, A.G. (2003). Enzyme kinetics: A modern approach. (New Jersey: John Wiley and Sons), pp. 50-52.

Matsuura, K., Ikoma, S., Sugiyama, M., Funauchi, M., and Sinohara, H. (1998). Amidolytic and peptidolytic activities of immunoglobulin G present in sera from patients with rheumatoid arthritis, Sjogren's syndrome and systemic lupus erythematosus. Immunol. *95*, 26-30.

Matsuura, K., and Sinohara, H. (1996). Catalytic cleavage of vasopressin by human Bence Jones proteins at the arginylglycinamide bond. Biol. Chem. *377*, 587-589.

Morelock, M.M., Rothlein, R., Bright, S.M., Robinson, M.K., Graham, E.T., Sabo, J.P., Owens, R., King, D.J., Norris, S.H., Scher, D.S., Wright, J.L., and Adair, J.R. (1994). Isotype choice for chimeric antibodies affects binding properties. J. Biol. Chem. *269*, 13048-13055.

Morrow, W.J., Williams, W.M., Whalley, A.S., Ryskamp, T., Newman, R., Kang, C.Y., Chamat, S., Kohler, H., and Kieber-Emmons, T. (1992). Synthetic peptides from a conserved region of gp120 induce broadly reactive anti-HIV responses. Immunol. *75*, 557-564.

Neshat, M.N., Goodglick, L., Lim, K., and Braun, J. (2000). Mapping the B cell superantigen binding site for HIV-1 gp120 on a VH3 Ig. International. Immunology *12*, 305-312.

Nishiyama, Y., Taguchi, H., Luo, J., Zhou, Y.-Z., Burr, G., Karle, S., and Paul, S. (2002). Covalent reactivity of a phosphonate monophenyl ester with serine proteinases: An overlooked feature of oxyanionic transition state analogs. Arch. Biochem. Biophys. *402*, 281–288.

Oleksyszyn, J., and Powers, J.C. (1994). Proteolytic enzymes: Serine and cysteine peptidases. In Methods in Enzymology. (New York: Academic Press), pp. 423-441.

Olshevesky, T.J., Helseth, E., Furman, C., Li, J., Haseltine, W., and Sodroski, J. (1990). Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. J. Virol. *64*, 5701-5707.

Patten, P.A., Gray, N.S., Yang, P.L., Marks, C.B., Wedemayer, G.J., Boniface, J.J., Stevens, R.C., and Schultz, P.G. (1996). The immunological evolution of catalysis. Science *271*, 1086-1091.

Paul, S., Planque, S., Zhou, Y.-X., Taguchi, H., Bhatia, G., Karle, S., Hanson, C., and Nishiyama, Y. (2003). Specific HIV gp120 cleaving antibodies induced by covalently reactive analog of gp120. J. Biol. Chem. *278*, 20429-20435.

Paul, S., Volle, D.J., Beach, C.M., Johnson, D.R., Powell, M.J., and Massey, R.J. (1989). Catalytic hydrolysis of vasoactive intestinal peptide by human autoantibody. Science *244*, 1158-1162.

Planque, S., Taguchi, H., Burr, G., Bhatia, G., Karle, S., Zhou, Y.-X., Nishiyama, Y., and Paul, S. (2003). Broadly distributed chemical reactivity of natural antibodies expressed in coordination with specific antigen binding activity. J. Biol. Chem. *278*, 20436-20443.

Pollard, S., Meier, W., Chow, P., Rosa, J., and Wiley, D. (1991). CD4-binding regions of human immunodeficiency virus envelope glycoprotein gp120 defined by proteolytic digestion. Proc. Natl. Acad. Sci. USA. *88*, 11320-11324.

Richieri, S.P., Bartholomew, R., Aloia, R.C., Savary, J., Gore, R., Holt, J., Ferre, F., Musil, R., Tian, H.R., Trauger, R., Lowry, P., Jensen, F., Carlo, D.J., Maigetter, R.Z., and Prior, C.P. (1998). Characterization of highly purified, inactivated HIV-1 particles isolated by anion exchange chromatography. Vaccine *16*, 119-129.

Roux, K.H., Strelets, L., Brekke, O.H., Sandlie, I., and Michaelsen, T.E. (1998). Comparisons of the ability of human IgG3 hinge mutants, IgM, IgE, and IgA2, to form small immune complexes: a role for flexibility and geometry. J. Immunol. *161*, 4083-4090.

Sampson, N.S., and Barton, P.A. (1991). Peptidic phophonylating agents as irreversible inhibitors of serine proteases and models of the tetrahedral intermediates. Biochemistry *30*, 22255-22263.

Shaw, D.C., Shultz, B.B., Ramsland, P.A., and Edmundson, A.B. (2002). Dealing with intractable protein cores: protein sequencing of the Mcg IgG and the Yvo IgM heavy chain variable domains. J. Mol. Recog. *15*, 341-348.

Shuster, A.M., Gololobov, G.V., Kvashuk, O.A., Bogomolova, A.E., Smirnov, I.V., and Gabibov, A.G. (1992). DNA hydrolyzing autoantibodies. Science *256*, 665-667.

Siliciano, R.F. (1996). The role of CD4 in HIV envelope-mediated pathogenesis. Curr. Top. Microbiol. Immunol. *205*, 159-179.

Sun, M., Gao, Q.-S., Kirnarskiy, L., Rees, A., and Paul, S. (1997). Cleavage specificity of a proteolytic antibody light chain and effects of the heavy chain variable domain. J. Mol. Biol. *271*, 374-385.

Taguchi, H., Burr, G., Karle, S., Planque, S., Zhou, Y.-X., Paul, S., and Nishiyama, Y. (2002). A mechanism-based probe for gp120-hydrolyzing antibodies. Bioorg. Med. Chem. Lett. *12*, 3167-3170.

Townsley-Fuchs, J., Kam, L., Fairhurst, R., Gange, S.J., Goodglick, L., Giorgi, J.V., Sidell, N., Detels, R., and Braun, J. (1996). Human immunodeficiency virus-1 (HIV-1) gp120 superantigen-binding serum antibodies. J. Clin. Invest. *98*, 1794-1801.

van Erp, R., Gribnau, T.C., van Sommeren, A.P., and Bloemers, H.P. (1991). Affinity of monoclonal antibodies. Interpretation of the positive cooperative nature of anti-hCG/hCG interactions. J. Immunol. Methods. *140*, 235-241.

Wentworth, A.D., Jones, L.H., Wentworth, P., Jr, Janda, K.D., and Lerner, R.A. (2000). Antibodies have the intrinsic capacity to destroy antigens. Proc. Natl. Acad. Sci. USA *97*, 10930-10935.

LEGENDS

Fig 1: Cleavage of biotinylated gp120 (Bt-gp120) by polyclonal human IgM and IgG preparations. A, Scatter plot of gp120 cleaving activity of IgM and IgG Ab fractions from 5 healthy humans. Ab combining site concentration 150 nM (decavalent IgM, 15 nM; bivalent IgG, 75 nM). Reaction conditions: 20 hours, 37°C, 100 nM Bt-gp120. Solid lines are means [IgM, 53.3 ± 25.4%; cleavage by IgG is below detection limit (<5%)]. *Inset,* Typical reducing SDS-electrophoresis (4-20% gels) results showing human serum IgM purified by affinity chromatography on immobilized anti-IgM Ab and stained with coomassie blue (lane 1) and peroxidase conjugated Abs to human μ chains (lane 2), κ chains (lane 3) and λ chains (lane 4). **B,** Streptavidin-peroxidase stained reducing SDS-gel lanes showing time-dependent cleavage of Bt-gp120 by pooled polyclonal human IgM. BC, Bt-gp120 incubated for 23 h in the absence of Abs. IgM, 50 nM; Bt-gp120, 100 nM.

Fig 2: gp120 cleavage by IgM subjected to denaturing gel filtration. Pooled human serum IgM purified by affinity chromatography on immobilized anti-μ Abs was subjected to cycles of denaturing gel filtration (cycle 1 ----; cycle 2 —; Superose 12 column) in 6 M guanidine hydrochloride. *Inset,* Streptavidin peroxidase stained SDS-gel lanes showing cleavage of Bt-gp120 (0.1 μM) by IgM (50 nM) obtained by denaturing gel filtration (lane 3) and control IgM analyzed without denaturation (lane 3). Reaction time, 16 h. Lane 1, Bt-gp120 incubated for 16 h with diluent instead of IgM.

Fig 3: gp120 cleavage by monoclonal IgM antibodies. A, Divergent catalytic activities of human monoclonal IgM; murine IgM clones 8702, 8704, 9008, 9010 and 9020 and polyclonal

IgM purified from pooled human and mouse sera. Biotinylated gp120, 0.1 μM. IgM, 17 nM. Data obtained by densitometry of streptavidin-peroxidase stained reducing SDS-gels. **Inset,** SDS-gel showing IgM Yvo stained with coomassie blue (lane 1), anti-human μ chain Ab (lane 2) and anti-κ chain Ab (lane 3). **B,** Gel filtration profile (Superose 12) of IgM Yvo without (---) and with (—) digestion with immobilized pepsin. *Inset,* Silver stained nonreducing (lane 1) and reducing (lane 2) SDS gels of the 55 kD Fab fragments. The higher and lower Mr fragments in the reducing gel correspond to the Fab heavy chain fragment and light chain component. **C,** streptavidin-peroxidase stained SDS-gels showing cleavage of biotinylated gp120 (0.1 μM) incubated with increasing concentrations of Fab Yvo for 48 h.

Fig 4: gp120 cleavage by monoclonal IgM Yvo. A, Time course of biotinylated gp120 (0.1 μM) cleavage by IgM Yvo (50 nM). Shown are streptavidin peroxidase stained reducing SDS-gel electrophoresis lanes. BC, Control lane showing Bt-gp120 incubated for 16 h without the IgM. The major biotinylated product is the 80 kD band. **B,** Coomassie blue stained SDS-gel lane showing the reaction mixture of gp120 (8.5 μM) with IgM Yvo (50 nM) (lane 1), IgM Yvo alone (lane 2) and gp120 alone (lane 3) incubated for 46 h. The 70 and 25 kD bands correspond to IgM heavy and light chains, respectively. Blots regions corresponding to 15.4-16.4 kD, 17.6 kD, 18 kD and 80 kD were subjected to N terminal sequencing in Table 1.

Fig 5: Selectivity of gp120 cleavage. Streptavidin peroxidase stained reducing SDS-polyacrylamide gels showing Bt-gp120, Bt-sEGFR, Bt-BSA and Bt-sCD4 incubated for 22 h in diluent or polyclonal human IgM (50 nM). Bt-protein, 0.1 μM.

Fig 6: gp120(421-431)-CRA inhibition of Yvo IgM gp120ase activity. A, Phosphonate diester analog of gp120 residues421-433 (gp120pep-CRA) and the haptenic phosphonate diester devoid of the gp120 peptide sequence (hapten CRA). **B,** Inhibition of IgM Yvo (50 nM) catalyzed Bt-gp120 (0.1 μM) by gp120pep-CRA. Incubation for15 hours. *Inset,* Strepavidin peroxidase stained SDS-gels showing Bt-gp120 incubated with IgM Yvo in the absence (lane 2) and presence of gp20pep-CRA (10 μM, lane 2). Lane 1, Control Bt-gp120 incubated in diluent.

Fig 7: Irreversible gp120pep-CRA binding by IgM. A, Comparative initial velocities for formation of hapten CRA adducts and gp120pep-CRA adducts by monoclonal IgM Yvo, 8704 and 9020 (●), IgM from pooled human serum (♦) and IgM from pooled mouse serum (■). Initial velocities were computed from progress curves for irreversible CRA binding by the Abs measured in duplicate, and represent the sums of intensities of the H chain-CRA and L chain-CRA bands. AAU, Arbitrary area units. Reaction conditions: IgM 150 nM; hapten CRA or gp120pep-CRA 10 μM. *Inset,* Strepavidin peroxidase stained reducing SDS-gels showing adducts of gp120pep-CRA (lane 1) and hapten CRA (lane 2) formed by polyclonal mouse IgM. **B,** Comparative initial velocities for formation gp120pep-CRA adducts by μ chains and κ/λ chains of monoclonal IgM Yvo, 8704 and 9020 (●), IgM from pooled human serum (♦) and IgM from pooled mouse serum (■). Reactions from panel A. **C,** Example of progress curve data. Shown are accumulation of gp120pep-CRA adducts and hapten CRA adducts with the L chain of IgM Yvo. Reactions as in panel A. *Inset,* Cut-outs of the L chain adduct bands at the indicated time points from strepavidin-peroxidase stained SDS-gels. **D,** Strepavidin peroxidase stained reducing SDS-gels showing adducts of gp120pep-CRA formed by polyclonal human IgM in the absence (lane 1) and presence of synthetic gp20(421-436) (500 μM, lane 2) and by IgM Yvo in the

absence (lane 3) and presence of synthetic gp20(421-436) (500 μM, lane 4). Reaction conditions as in Panel A. Incubation for 4.5 hours.

Fig 1

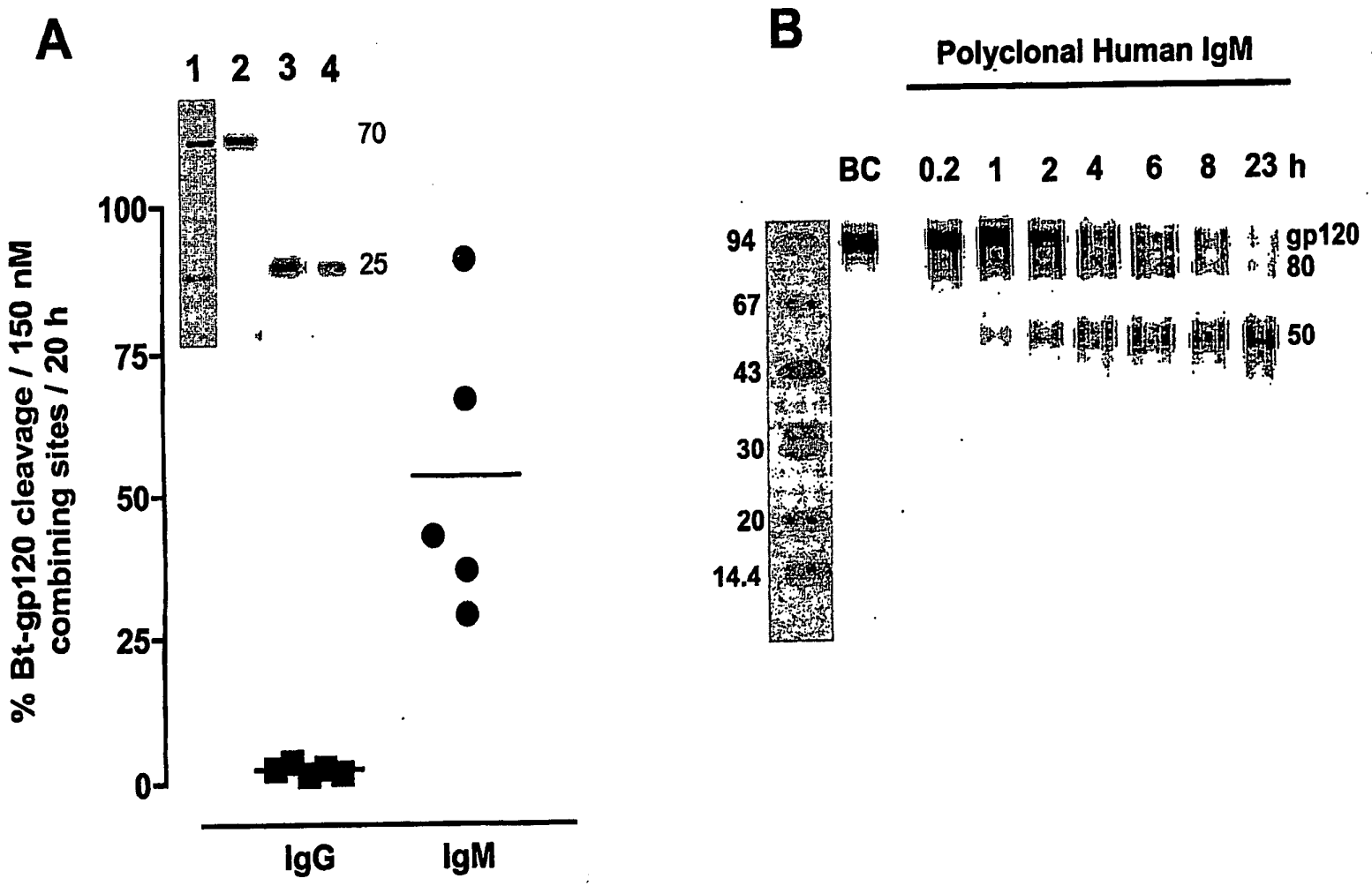

A
1 2 3 4
70
25
100
75
50
25
0
% Bt-gp120 cleavage / 150 nM combining sites / 20 h
IgG
IgM
B
Polyclonal Human IgM
BC 0.2 1 2 4 6 8 23 h
94
67
43
30
20
14.4
gp120
80
50

Fig 2

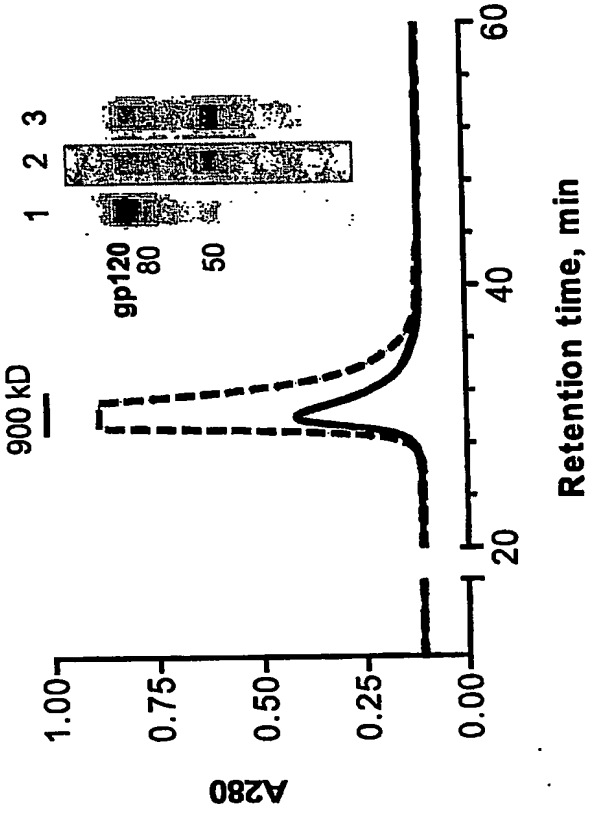

900 kD
1 2 3
gp120
80
50
A280
1.00
0.75
0.50
0.25
0.00
20
40
60
Retention time, min

Fig 3

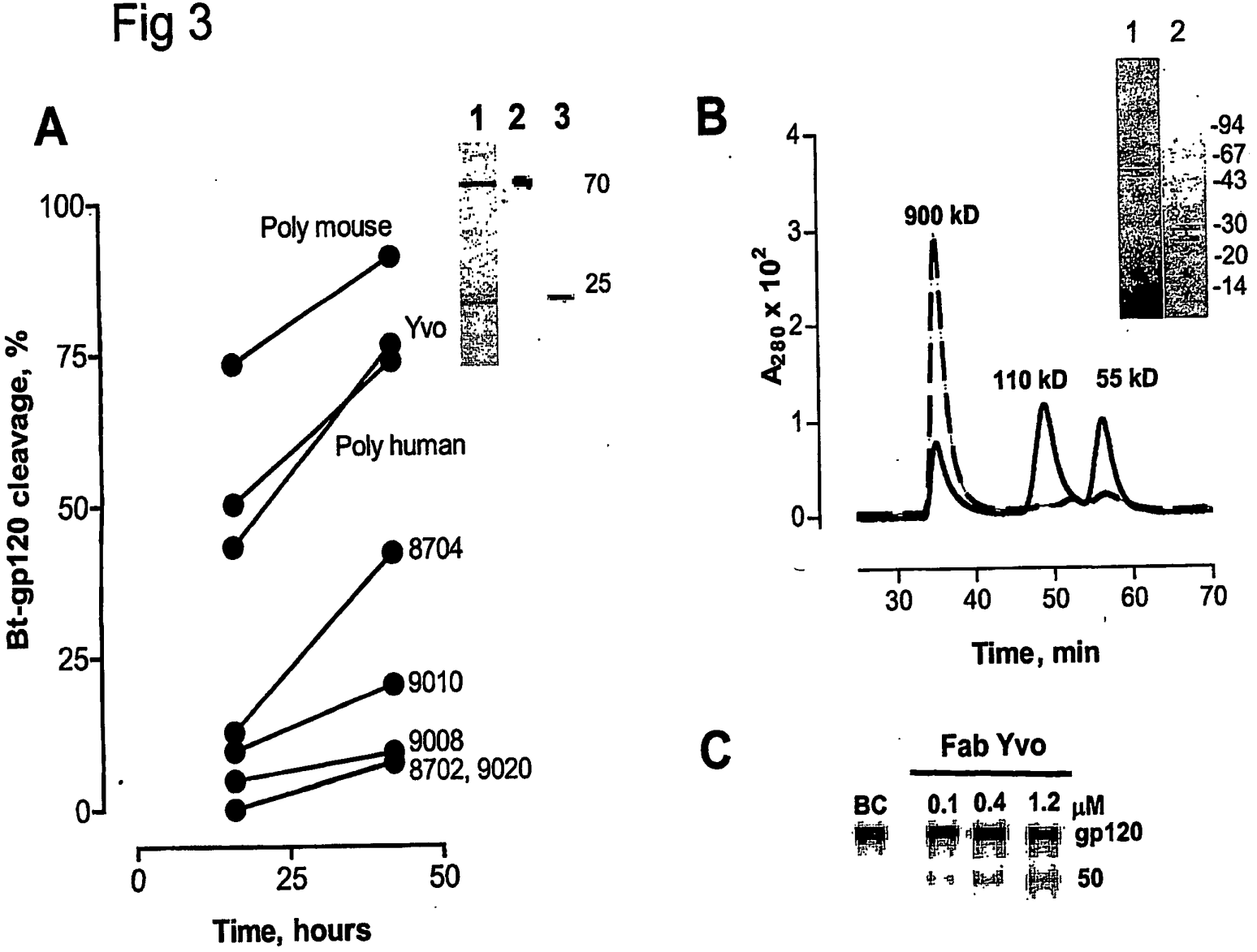

A
1 2 3
70
25
100
75
50
25
0
Bt-gp120 cleavage, %
Poly mouse
Yvo
Poly human
8704
9010
9008
8702, 9020
0
25
50
Time, hours
B
1 2
-94
-67
-43
-30
-20
-14
900 kD
110 kD
55 kD
$A_{280} \times 10^2$
4
3
2
1
0
30
40
50
60
70
Time, min
C
Fab Yvo
BC
0.1
0.4
1.2 μM
gp120
50

Fig 4

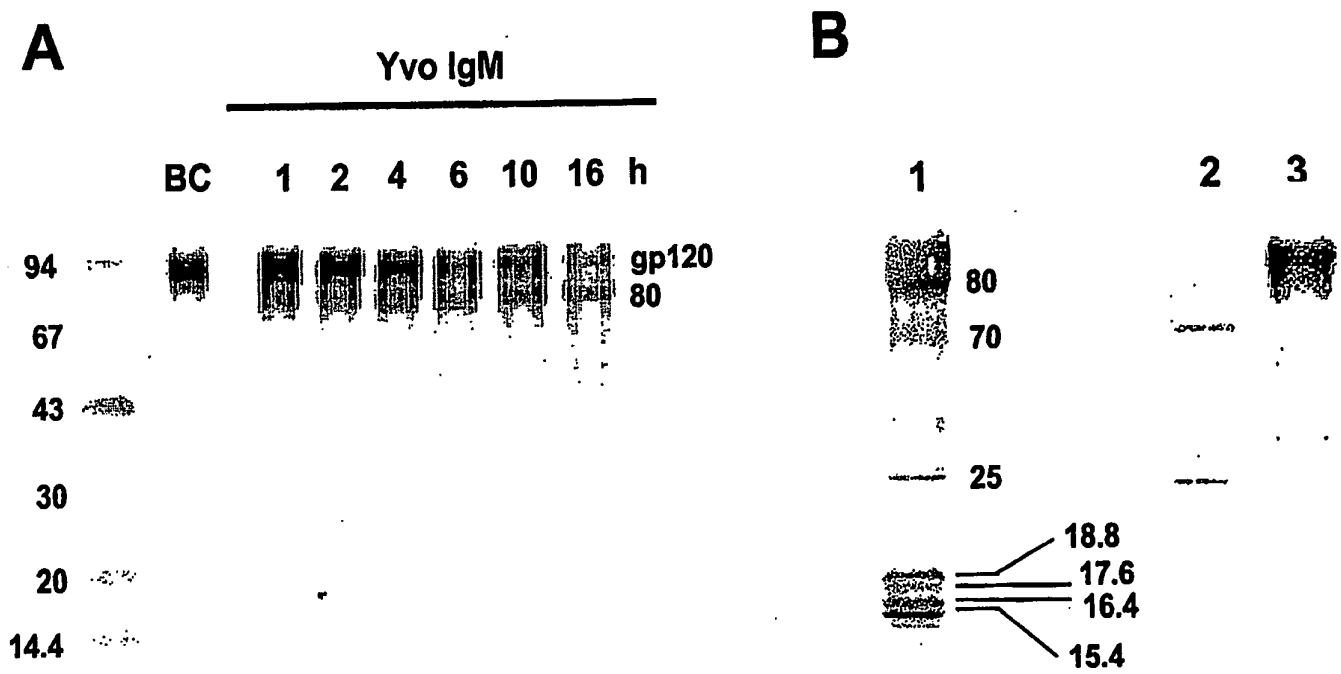

A
Yvo IgM
BC
1
2
4
6
10
16
h
94
67
43
30
20
14.4
gp120
80
B
1
2
3
80
70
25
18.8
17.6
16.4
15.4

Fig 5

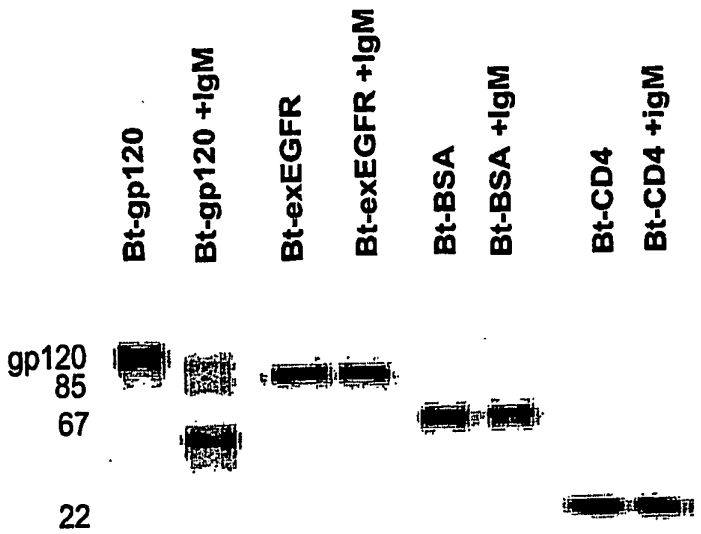
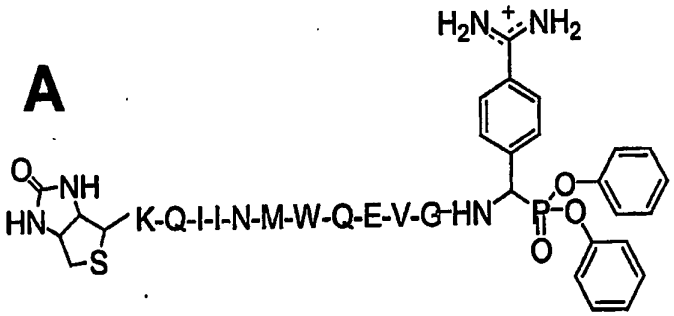
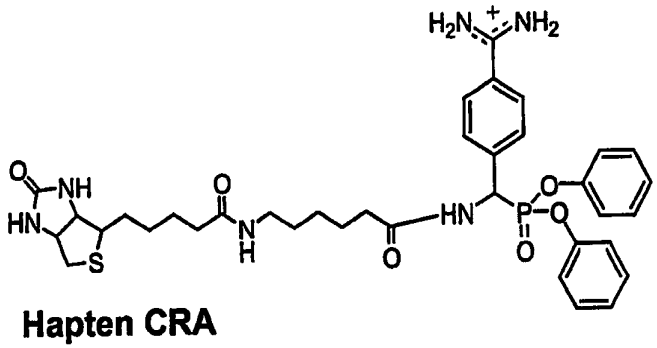

Bt-gp120
Bt-gp120 +IgM
Bt-exEGFR
Bt-exEGFR +IgM
Bt-BSA
Bt-BSA +IgM
Bt-CD4
Bt-CD4 +igM
gp120
85
67
22

Fig 6


A
K-Q-I-I-N-M-W-Q-E-V-G-HN
gp120pep-CRA


Hapten CRA

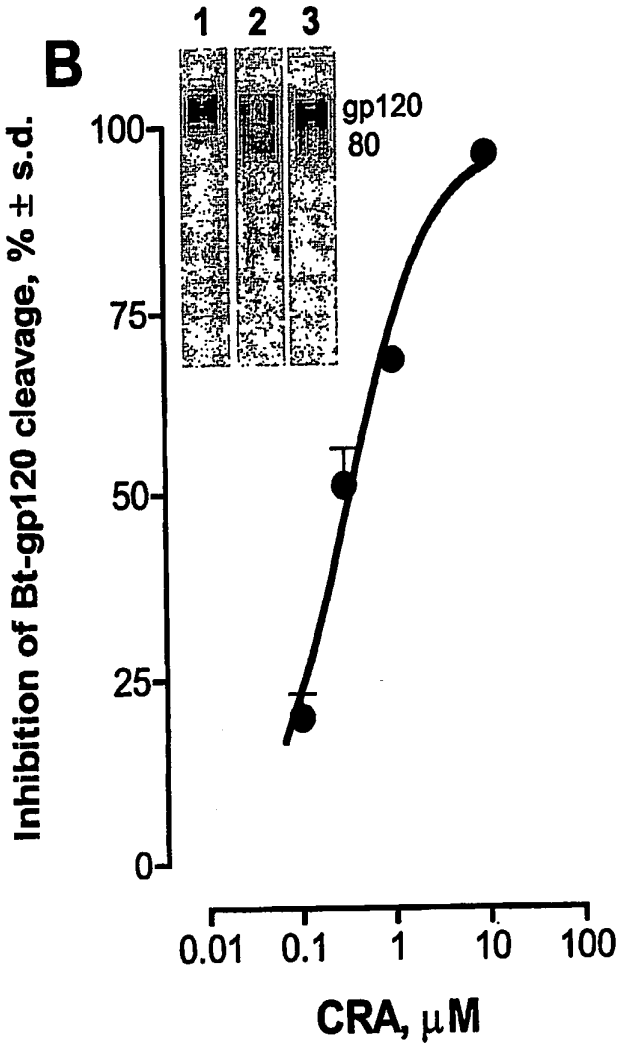
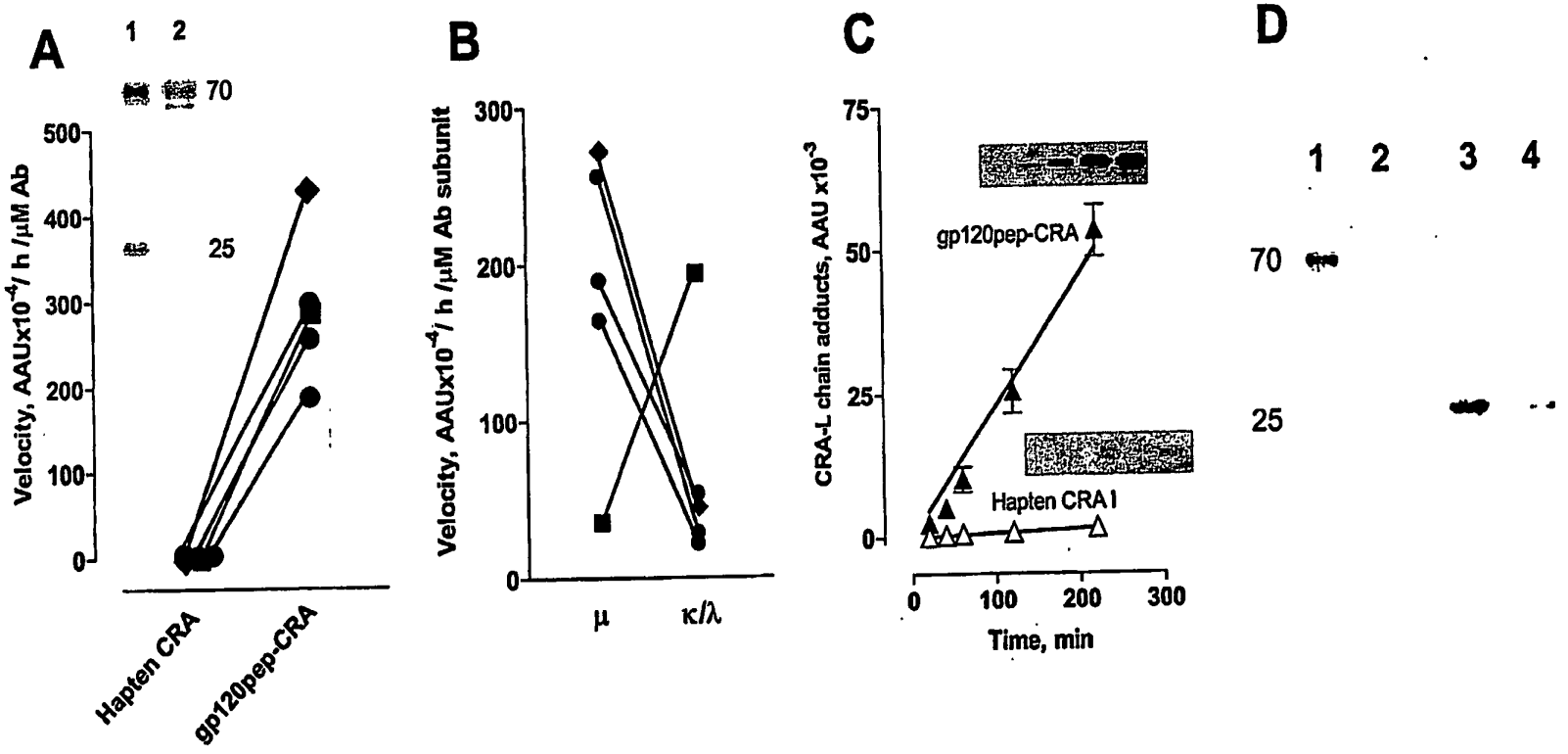

B
1 2 3
gp120
80
Inhibition of Bt-gp120 cleavage, % ± s.d.
100
75
50
25
0
0.01 0.1 1 10 100
CRA, μM

Fig 7


A
1 2
70
25
Velocity, AAUx10⁻⁴/ h /μM Ab
500
400
300
200
100
0
Hapten CRA
gp120pep-CRA
B
Velocity, AAUx10⁻⁴/ h /μM Ab subunit
300
200
100
0
μ
κ/λ
C
CRA-L chain adducts, AAU x10⁻³
75
50
25
0
gp120pep-CRA
Hapten CRA I
0
100
200
300
Time, min
D
1 2 3 4
70
25

Product, kD	Amino acid sequence[a]	Cleavage site
80	I(2.3), P(1.7), G(1.2), E(1.9), K(0.8), L(1.5), X, V(1.4), T(1.2), V(1.4)	N.I.
18.8	I(8.4), P(6.3), G(3.7), E(5.7), K(4.0), L(5.1), W(0.4), V(4.8), T(3.0), V(4.8)	N.I.
17.6	A(1.7), M(0.6), Y(0.8), A(0.9), P(0.5), P(0.5), I(0.4), E(0.4), G(0.4), Q(0.3)	K^{432} - A^{433}
15.4-16.4	A(9.4), M(5.3), Y(6.7), A(8.7), P(6.3), P(4.6), I(4.8), E(3.8), G(2.4), Q(3.5) I(1.7), P(0.8), G(1.6), E(1.3), K(0.4), L(1.1), W(0.1), V(1.1), T(1.0), V(1.2)	K^{432} - A^{433} N.I.

Table 1: N terminal sequences (10 residues) of gp120 fragments generated by IgM Yvo. Reaction conditions: gp1208.5 μM, IgM 50 nM, 46 h. N.I., not identifiable; however, identification of the 15.4-16.4 and 18.8 kD fragments with N-termini corresponding to gp120 residues 1-10 indicates two cleavage sites located in the N-terminal half of the protein. X, unidentified amino acid.

[a] Values in parentheses indicate recovery of the amino acids in pmol.

Antibody	K_d, M	k_{cat}, min^{-1}	k_{cat}/K_d, $M^{-1}min^{-1}$
IgM Yvo	1.3×10^{-6}	0.04 ± 0.002	2.8×10^4
IgM polyclonal human	31.0×10^{-6}	2.14 ± 0.03	6.8×10^4
IgM 8704	7.4×10^{-6}	0.12 ± 0.05	1.6×10^4

Table 2: Apparent kinetic parameters for IgM catalyzed biotinylated gp120 cleavage. Increasing gp120 concentrations (2, 1, 0.5, 0.25, 0.125 μM) treated in duplicate with IgM (50 nM) for a sufficient length of time to yield gp120 cleavage levels in the measurable, linear phase of the reaction. See text for method of k_{cat} and K_d computation.

Table 2: Apparent kinetic parameters for IgM catalysis. Substrate, Glu-Ala-Arg-AMC (25-600 μM); IgM, 5 nM. Correlation coefficients for fits to the Michaelis-Menten equation were ≥ 0.96 in every case.

Antibody	K_m, M	k_{cat}, mol/mol Ab/ min
IgM, murine serum	$120 \pm 22 \times 10^{-6}$	2.1 ± 0.1
IgM 9010	$144 \pm 15 \times 10^{-6}$	1.9 ± 0.1
IgM 9020	$154 \pm 28 \times 10^{-6}$	0.9 ± 0.1
IgM, human serum	$120 \pm 11 \times 10^{-6}$	2.8 ± 0.1
IgG c23.5*	0.34×10^{-9}	8×10^{-4}

* From ref 6. Substrate, VIP